DOCTOR OF PHILOSOPHY

Modulation of Skeletal Muscle Insulin Sensitivity and SNAT2 Amino Acid Transporter Expression by Fatty Acid Availability

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Modulation of Skeletal Muscle Insulin Sensitivity and SNAT2 Amino Acid Transporter Expression by Fatty Acid Availability

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Abbreviations

4EBP1	eIF4E-binding protein 1
α	Alpha
AAR	Amino acid response
AARE	Amino acid response element
ACC	Acetyl CoA carboxylase
ACP	Acyl carrier protein
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP activate protein kinase
ARA	Arachidonic acid
AS160	Akt substrate of 160 kDa
ASCT2	ASC-amino acid transporter 2
ATF4	Activating transcription factor 4
ATGL	Adipose triacylglycerol lipase
ATP	Adenosine triphosphate
β	Beta
BAD	Bcl-2 associated death promoter
Bcl2	B-cell lymphoma 2
BH3	Bcl-2 homology domain 3
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CACT	Carnitine acylcarnitine translocase
CARE	CCAAT-enhancer binding protein-activating transcription factor (C/EBP-ATF) response elements
CAT-1	Cationic amino acid transporter 1
Cdc2	Cyclin dependent kinase
CDC25	Cell division cycle 25
CDKI	Cyclin-dependent kinase inhibitor
CHOP	C/EBP homology protein
CMV	Cytomegalo virus
CO_2	Carbon dioxide
CoA	Coenzyme A
CPT	Carnitine palmitoyl transferase
CREB	Cyclic AMP Response Element-Binding protein
Δ/δ	Delta
DAG	Diacylglycerol
DGAT	Diacylglycerol acyltransferase
DHA	Docosahexaenoic acid
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPS	Deoxynucleotide triphosphates
DPM	Disintegrations per minute
DRP1	Dynamin related protein 1
DUSP	Dual-specificity MAPK phosphatase
ε	Epsilon
η	Eta
EBSS	Earle’s balanced salt solution

EDTA	Ethylenediaminetetraacetic acid
eIF	Eukaryotic initiation factor
Elovl	Elongation of very-long-chain fatty acid
EPA	Eicosapentaenoic acid
ER	Endoplasmatic reticulum
ERK	Extracellular regulated kinase
Etomoxir	Ethyl-2-[6-(4-chlorophenoxy) hexyl]-oxirane-2-carboxylate
FABP	Fatty acid binding protein
FAD	Fatty acid desaturase
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FFA	Free fatty acid
FoxO	Forkhead box-O protein
FRET	Fluorescence resonance energy transfer
γ	Gamma
g	Acceleration due to gravity
g	Grams
Gab1	Grb2-associated binder-1
GADD34	Growth arrest and damage-inducible 34
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GCN2	General control non-responsive 2
GLT1	Glial Glutamate Transporter 1
GLUT	Glucose transporter
Grb2	Growth factor receptor-bound protein 2
GRP1	General receptor of phosphoinositides 1
GP	Glycerol-phosphatase
GS	Glycogen synthase
GSK3	Glycogen synthase kinase
GST	Glutation S-transferase
GTP	Guanosine triphosphate
h	Hours
H^+	Hydrogen ion (or hydron)
H_2O	Water
HBS	Hepes buffered saline
HL	Hepatic lipase
HSL	Hormone sensitive lipase
IkB	Inhibitor of kappa B
IKK	Inhibitor of kappa B kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IR	Insulin receptor
IRES	Internal ribosome entry site
IRS1	Insulin receptor substrate 1
JNK	c-jun N-terminal kinase
k	Kappa
K	Lysine
K^+	Potassium
l	litre
L	Leucine

LAT-1	L-type amino acid transporter 1
LCMT-1	Leucine carboxyl methyl transferase-I
LPL	Lipoprotein lipase
LOA	Linoleic acid
µ	Micro (10^{-6})
m	Milli (10^{-3})
M	Molar
MAFbx	Muscle Atrophy F-Box
MAG	Monoacylglycerol
MAPK	Mitogen activated protein kinase
MeAIB	2-methylaminoisobutyric acid
MEFi	Mouse embryonic fibroblast immortalised
MEK	Mitogen-activated extracellular-regulated kinase
MEM	Minimal essential medium
min	Minute
M-MLV	Moloney Murine Leukemia Virus
MND	Minidiscs (*Drosophila melanogaster* gene)
mRNA	Messenger ribonucleic acid
mTOR	Mammalian taget of rapamycin
mTORC1	Mammalian target of rapamycin (mTOR) complex 1
mTORC2	Mammalian target of rapamycin (mTOR) complex 2
MUFA	Monounsaturated fatty acid
MuRF-1	Muscle RING Finger-1
n	Nano (10^{-9})
Na^+	Sodium
Nedd4.2	Neural precursor cell expressed, developmentally down-regulated
4-2	
NEFa	Non-esterified fatty acid
NFkB	Nuclear factor kappa B
ω	Omega
O_2	Oxygen
OA	Oleic acid
P90RSK	90 kDa Ribosomal protein S6 Kinase
P70S6K	p70 ribosomal protein S6 kinase
PA	Palmitate
Path	Pathetic (*Drosophila melanogaster* gene)
PCR	Polymerase chain reaction
PDHK	Pyruvate dehydrogenase kinase
PDK1	3-phosphoinositide-dependent protein kinase-1
PEI	Polyethilenimine
PGC1α	PPAR-gamma-co-activator-1α
PH	Pleckstrin domain
PHLLP	PH-domain leucine-rich repeat protein phosphatase
PI3K	Phosphoinositide-3-kinase
$PI(3,4,5)P_3$	Phosphatidylinositol 3,4,5-trisphosphate
PKB/Akt	Protein kinase B
PKC	Protein kinase C
PME-1	PP2A methylesterase-1
PP2A	Protein phosphatase 2 A
PPAR	Peroxisome proliferator-activated receptor

PTB	Phosphotyrosine-binding
PTEN	Phosphatase and tensin homolog
PTS	Phosphoenol pyruvate- carbohydrate phosphotrasferase system
PUFA	Polyunsaturated fatty acid
PVDF	Polyvinylidene difluoride
qPCR	Quantitative PCR
Raf	Rapidly phosphorylated fibrosarcoma
Rag	Ras-reated GTPase
Rheb	Ras homologue enriched in brain
RNA	Ribonucleic acid
RNAi	RNA interference
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
s	Second
S	Serine
SCD	Steraoyl-CoA desaturase
SDS-PAGE	Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis
SFA	Saturated fatty acid
SH2	Src-homology 2
SHP-2	Src-homology-2-containing protein
shRNA	Short hairpain interfering RNA
SIRTI1	Sirtuin 1
Slif	Slimfast (*Drosophila melanogaster* gene)
SNAT2	Sodium-coupled neutral amino acid transporter 2
SOS	Son of sevenless protein
SPT	Serine palmitoyl transferase
SREBP-1	Sterol regulatory element binding protein-1
θ	Teta
T	Threonine
TAG	Triacylglycerol
Tap42/α4	Type 2A-associated protein of 42 kDa
TBST	Tween 20/Tris buffered saline
TCA	Trichloroacetic acid
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TRB3	Tribbles-3
TSC1/2	Tuberous sclerosis complex 1/2
Ubxd8	Ubiquitin like (Ubx)-domain containing protein 8
UDP	Uridine diphosphate
UFA	Unsaturated fatty acid
UPS	Ubiquitin proteasomal system
UTR	Untranslated region
VLDL	Very low density lipoproteins
Vps34	Vacuolar protein sorting 34
ζ	Zeta
Y	Tyrosine

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Declarations

I hereby declare that I am the sole author of this thesis and have consulted all the references cited, whether in full or in abstract form. The work reported here was conducted by myself, with the following exceptions:
Chapter 3. Human myotubes experiments were performed by Rima Hage Hassan, Université Pierre et Marie Curie, Paris.
Chapter 4. The experiments presented in Figure 4.9A and 4.14A were performed respectively by Dr Emma Cwiklinski and Dr Russel Hyde, Division of Cell Signalling and Immunology, University of Dundee.
This thesis has not, in whole, or in part, been previously submitted for a higher degree.

Signed.....
Francesca Nardi

Date.....

I certify that Francesca Nardi has spent the equivalent of at least nine terms in research work within the College of Life Sciences, Univeristy of Dundee, and that she has fulfilled the conditions of the Ordinance General 14 of the University of Dundee and is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

Signed.....
Hari Hundal
Professor of Molecular Physiology, University of Dundee

Date.....

Abstract

Skeletal muscle accounts for ~75% of whole body insulin-stimulated glucose uptake and contains the largest intracellular pool of free amino acids and consequently exerts a major influence on whole body carbohydrate and protein metabolism. Muscle also represents an important site for fat oxidation especially after fasting, but sustained over-supply of fatty acids under well nourished conditions can deleteriously affect signalling processes regulating carbohydrate and protein metabolism. In particular, there is mounting evidence that elevated concentration of plasma non-esterified saturated fatty acids (SFAs) leads to insulin resistance, whereas increased content of monounsaturated (MUFAs) or polyunsaturated (PUFAs) fatty acids confers a protective effect and/or improves insulin sensitivity. However the molecular mechanisms by which unsaturated fatty acids exert this beneficial effect are still unknown. To address this issue, in the first part of the study presented in this thesis I have examined the effect of the MUFA oleic acid and the PUFA linoleic acid upon proximal insulin signalling events in L6 rat skeletal muscle cells. I demonstrate that chronic incubation of L6 myotubes with oleic and linoleic acid significantly enhanced insulin stimulated activation of the mitogen-activated protein (MAP) kinase (ERK1/2) and PI3K/Akt signalling pathways and counters the detrimental effect of the SFA palmitate. Importantly, I show that the observed improvements in insulin action are not due to augmented IRS1/PI3K activation, but rather involve suppressing the action of protein phosphatase 2A (PP2A) towards Akt and ERK1/2.

Although the effect of SFAs and UFAs upon insulin signalling has been amply documented, very little is known about how fatty acids may affect amino acid

transport. Interestingly, previous work from our lab has demonstrated that ceramide, a derivative of the saturated fatty acid palmitate, can suppress the basal and insulin-stimulated activity of the SNAT2 amino acid transporter by preventing its translocation to the plasma membrane, an effect that it also exerts on the insulin-regulated glucose transporter, GLUT4. The second part of this thesis thus reports studies that focus on the effects of palmitate, linoleic and oleic acid on SNAT2 expression/activity in L6 rat skeletal muscle cells and HeLa cells. SNAT2 is one of the most abundant and highly regulated short chain neutral amino acid transporter known to be expressed in mammalian cells. The transporter is extensively regulated in response to nutrient/osmotic cues. Extracellular amino acid deprivation or hyperosmotic shock, for example, induce SNAT2 abundance in the plasma membrane by a process known as “adaptive regulation”, which involves increased expression of SNAT2 and its translocation from an intracellular pool to the plasma membrane where it is maintained in a stable and active state. The results presented herein show that linoleic acid and palmitate significantly impair the SNAT2 adaptive response in a dose and time-dependent manner by substantially reducing SNAT2 membrane protein and consequent activity. The failure to upregulate cellular SNAT2 content in response to amino acid limitation is not due to suppression in SNAT2 gene transcription but to a loss in the abundance of the mature/glycosylated form of SNAT2 at the membrane. Linoleic acid induces this effect by enhancing proteasomal degradation of SNAT2. Previous work has in fact shown that SNAT2 stabilization and turnover at the plasma membrane involves the ubiquitin–proteasomal pathway and that its ubiquitination is mediated by the E3 ligase Nedd4.2. My work reveals that use of a proteasomal inhibitor, MG132,

negates the loss in SNAT2 triggered by linoleic acid. Intriguingly, however, shRNA silencing of Nedd4.2 does not protect against fatty-induced proteasomal loss of SNAT2 suggesting that SNAT2 ubiquitination is likely to involve an E3 ligase other than Nedd4.2. In contrast, the effect of palmitate upon SNAT2 protein abundance in the plasma membrane is due to transporter internalisation, promoted by its derivative ceramide, and potential palmitate-induced lysosomal degradation of the “surface” mature form of SNAT2.

Publications

Some of the results in Chapter 3 and 4 have been presented in the following publications:

Nardi F, Lipina C, Magill D, Hage Hassan R, Hajduch E, Gray A, & Hundal HS. (2014) **Enhanced Insulin Sensitivity Associated with Provision of Monounsaturated and Polyunsaturated Fatty Acids in Skeletal Muscle Cells Involves Counter-modulation of PP2A.** *PLoS One*. 2014, **9** (3):e92255.

Nardi F, Hoffmann T, Stretton C, Cwiklinski E, Taylor PM and Hundal HS (2014) **Proteasomal modulation of cellular SNAT2 (SLC38A2) abundance and function by unsaturated fatty acid availability.** *J.Biol.Chem*. (published online February 4, 2015).

Chapter 1

Introduction

1.1 Overview

All organisms are faced with the constant challenge of surviving in stressful situations. Nutrient availability is temporally and spatially variable therefore and consequently organisms have developed mechanisms to store nutrients if in excess, in order to make them available when they become limited. Nutrients are stored in the body as carbohydrates, lipids and proteins and their release and utilisation are tightly regulated.

Glucose represents the main source of energy of the body. During the postprandial period, blood glucose rises and induces the release of insulin, which regulates uptake of glucose into key tissues such as muscle and fat (Flatt, 1995). Since oxidation accounts for ~10 g glucose/h, it follows that most of the glucose taken up in such tissues is either stored in the form of glycogen or directed towards lipid synthesis and storage (Flatt, 1995). Under low-glucose levels, fatty acids can be used as source of energy by most body tissues. Fat cells that make up the adipose tissue are specialised in the storage of fatty acids as triacyglycerols (TAGs) (Flatt, 1995). During the fasting state, glycogen is the first energy store to be depleted, followed by TAGs and only after a prolonged period of starvation is body protein degraded for release of amino acids. Amino acids can be used as an alternative energy source once circulating levels of both glucose and fatty acids become low. Unlike glycogen or triacylglycerol, proteins do not primarily function as energy stores but perform distinct structural or functional roles within cells. The fraction of total dietary energy provided by proteins is relatively small and the body spontaneously maintains its content by regulating amino acid oxidation and uptake. Modulating cellular uptake, synthesis and metabolism of amino acids as well as synthesis

and degradation of proteins within cells collectively influence the cellular amino acid pool size (Flatt, 1995).

Skeletal muscle constitutes ~50% of lean body mass and consequently skeletal muscle metabolism strongly influences the metabolic budget of the whole body. Under insulin stimulation, this tissue is responsible for ~75% of whole body glucose disposal, accounting for 3-4 fold greater glycogen storage than that in the liver (Flatt, 1995). In addition, skeletal muscle is the major bodily store of free amino acids and the main site of gluconeogenic amino acid synthesis (Frayn, 1996). During periods of muscular contraction, skeletal muscle is the principal site of nutrient consumption and therefore the transport of metabolic substrates, such as glucose and certain amino acids, is rapidly upregulated (Zorzano et al., 2000). Importantly, during prolonged levels of exercise most energy needs come from use of fat as an energy fuel rather than from carbohydrates. Therefore, skeletal muscle is a tissue with a complex metabolic network, in which exercise and hormones regulate the uptake and metabolism of glucose, fatty acids and amino acids.

1.2 Overview on insulin, glucose and fatty acids

1.2.1 Insulin

Insulin is a peptide hormone produced by pancreatic β-cells and responsible for the regulation of diverse processes including glucose and fat metabolism. It consists of a 21 amino acid α chain and a 30 amino acid β chain, linked by disulfide bonds. The mammalian insulin gene is exclusively expressed in β cells of the endocrine pancreas and encodes for a single 110 amino acid polypeptide chain precursor, preproinsulin (Chan et al., 1976), then cleaved to produce

proinsulin. Proinsulin is packed into secretory vesicles where it matures into active insulin (Halban, 1991). Glucose enters into pancreatic β-cells *via* the GLUT2 glucose transporter, is metabolised and ATP generated from its metabolism promotes closure of K_{ATP} channels that result in plasma membrane depolarisation and subsequent activation of voltage-gated Ca^{2+} channels. The increase in intra-cellular Ca^{2+} levels stimulates the fusion of insulin-containing granules with the β-cell plasma membrane and consequent release of insulin (Newgard and McGarry, 1995). Upon its release, insulin plays a critical role in regulating metabolism of glucose, protein and fat. One of its primary functions is to stimulate glucose uptake in key target tissues, such as skeletal muscle and adipose tissue. Whilst adipose tissue plays a minor role with respect to insulin-induced glucose disposal, skeletal muscle represents the predominant “sink” for whole body glucose disposal (Saltiel and Kahn, 2001). Whilst insulin does not increase hepatic glucose uptake the hormone stimulates the storage of glucose as glycogen in liver, while preventing its production and release by inhibition of gluconeogenesis and glycogenolysis (Pilkis and Granner, 1992). In adipose tissue, insulin suppresses the release of non-esterfied fatty acids and promotes their esterification and storage as TAG (Frayn, 2002).

1.2.2 Glucose

Glucose is a key metabolite and a major fuel source in mammals. It is a cyclic aldose monosaccharide and can be found in two different stereoisoforms, D-glucose and L-glucose, of which only the first one can be utilised by mammals as a source of energy and a metabolic intermediate (Berg, 2002). In addition to dietary glucose the hexose can also be synthesised in liver and kidneys from non-carbohydrate intermediates, such as pyruvate, lactate and glycerol through

a process known as gluconeogenesis (Stumvoll et al., 1998). Moreover, glucose can be obtained by breaking down glycogen stores in liver and skeletal muscle by glycogenolysis. Glucose uptake differs among tissues depending on glucose availability and the metabolic need of each tissue. The two main ways by which glucose can enter into cells is *via* facilitative transport and secondary active transport. Facilitative transport is driven by the downward chemical gradient of glucose across the plasma membrane and is mediated by members of the GLUT family of transporters. Among these, GLUT4 is the main insulin-sensitive member of this family and is uniquely expressed in adipose tissue, skeletal and cardiac muscle (Wood and Trayhurn, 2003). While most cells take up glucose by facilitative transport, epithelial cells of the small intestine and kidney proximal convoluted tubule absorb glucose against its electrochemical gradient by a secondary active transport mechanism, mediated by the family of Na+/glucose cotransporters (gene symbol SLC5A, protein symbol SGLT) (Wood and Trayhurn, 2003; Wright, 2001).

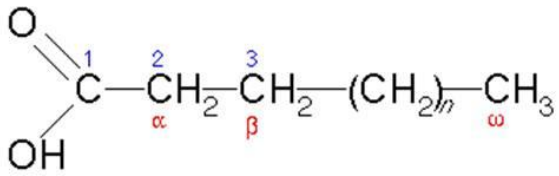
Once taken up into cells, glucose can enter three main metabolic processes: 1) oxidation, 2) storage and 3) lactate production. How glucose is directed to these different processes depends mainly on tissue necessity (Berg, 2002). Since neuronal tissues lack the ability to oxidise fatty acids or amino acids without undergoing adverse structural and functional changes, most of the glucose taken up by the brain is used as fuel and therefore oxidised (Maher et al., 1994). Skeletal muscle instead mainly channels glucose into lactate production during fasting, while promoting its storage as glycogen after insulin release (Zierler, 1999).

1.2.3 Fatty acids

Fatty acids are hydrocarbon chains that terminate with carboxylic acid groups. Carbon atoms of the fatty acids are numbered starting at the carboxyl terminus. Carbon atoms 2 and 3 are also called α and β respectively, while methyl carbon atom at the distal end of the chain is known as ω-carbon atom (Fig 1.1 A). The aliphatic chain contains an even number of carbon atoms, usually between 4 and 14, with 16- and 18- carbon-long fatty acids being the most common. The aliphatic chain can be saturated or contain one or more double bonds, which define the difference between saturated, monounsaturated and polyunsaturated fatty acids (Fig 1.1 B). The configuration of the double bonds in most unsaturated fatty acids is *cis*, and if more double bonds are present at least one methylene group separates them. Chain length and degree of saturation affect the biophysical features of fatty acids such as their melting point or their absorption (Berg, 2002). As such, shorter chain length and more unsaturation enhance the fluidity of the fatty acid and its derivatives (Berg, 2002). Most fatty acids can be synthesised in the liver but two of them, linoleic acid (C18:2) and α-linolenic acid (C18:3), are considered “essential” as they cannot be synthesised and must be supplemented in the diet (Plourde and Cunnane, 2007). Fatty acids are generally stored as TAG, a lipid comprised of a glycerol backbone to which three fatty acids are esterified. TAGs circulate in the plasma as part of lipoproteins and are also stored in cells as neutral lipid droplets. The tissues that typically store TAG and hydrolyse it, either for internal oxidation or export, are adipose tissue, skeletal muscle and liver (Coleman and Mashek, 2011). The main source of dietary fatty acids is in the form of TAG. In the intestinal lumen, TAG is broken down by pancreatic lipase to monoacylglycerol

and free fatty acids, which aggregate with bile acid and other lipids to form structures known as micelles (Carlier et al., 1991). Micelles are transported from the intestinal lumen into mucosal cells, where free fatty acids and monoacylglycerol are re-packed as TAG and combined with cholesterol, lipoproteins and other lipids into particles called chylomicrons. Chylomicrons are the largest lipoprotein particles and enter into the blood circulation to deliver TAG-fatty acids to the peripheral tissues. TAG and fatty acids are absorbed by tissues *via* lipoprotein lipase (LPL), an enzyme expressed on the luminal side of the capillary endothelial cells of adipose tissue, skeletal and cardiac muscle cells. LPL binds and hydrolyses chylomicrons releasing fatty acids and monoacylglycerol for take up by these tissues. The remnant part of the lipoprotein particle is taken up by the liver, which expresses hepatic lipase (HL), an enzyme that acts on smaller particles than LPL (Frayn et al., 2006). When the body has excess carbohydrate availability, the liver has the ability to synthesise new fatty acids from glucose and store them as TAG (Hellerstein et al., 1996). During fasting, the cytosolic TAG pool undergoes lipolysis to generate fatty acids, which are re-assembled in very low density lipoproteins (VLDL) and released into blood circulation to reach other tissues (Frayn et al., 2006). Adipose tissue is the body's largest energy reservoir and represents the main target of circulating chylomicrons and VLDL. In particular, during the postprandial state insulin stimulates the uptake of excess circulating TAG into the adipose tissue. Moreover, it induces uptake of glucose, whose metabolism provides a source of glycerol-3 phosphate necessary for esterification of free fatty acids and generation of TAG. During energy deprivation, enzymes such as hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) regulate

(A)



(B)

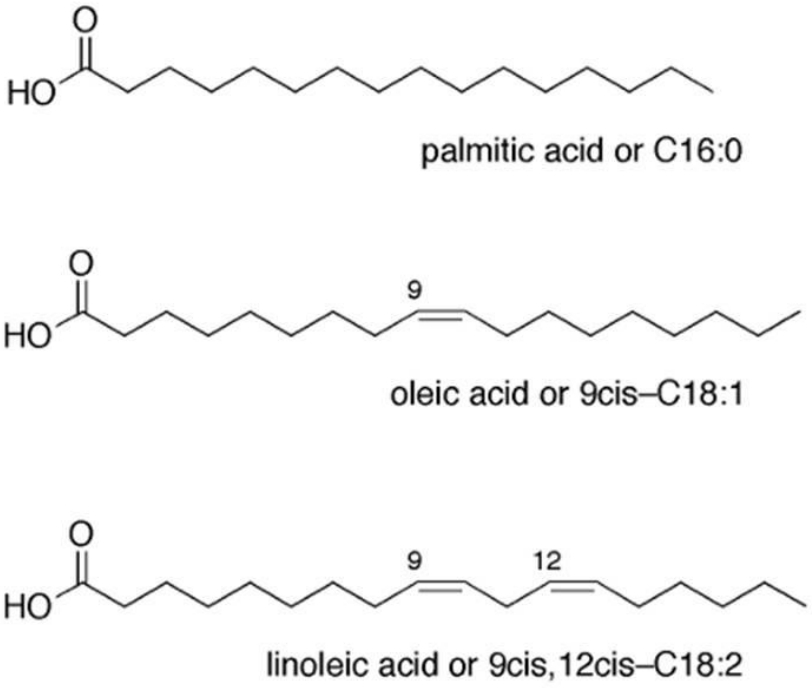


Figure 1.1 General fatty acid structure and structures of the most common fatty acids.

(A) Numberig system of fatty acid structure. **(B)** Palmitate is a 16-carbon, saturated fatty acid, oleic acid is an 18-carbon fatty acid with a single *cis* double bond and linoleic acid is an 18-carbon fatty acid with a two *cis* double bonds. Modified from (Chatgilialoglu, 2010).

the lipolysis of stored TAG and consequent release to the blood circulation as glycerol and non esterified free fatty acid (NEFA), which become available for uptake by other tissues such as liver or skeletal muscle (Lafontan and Langin, 2009). Skeletal muscle can take up fatty acids either from circulating TAG *via* action of LPL, or from the plasma NEFA pool. NEFAs are insoluble and those transported in plasma are bound to albumin. The dissociation of the fatty acids from albumin represents the first step in their uptake, which is then mediated by fatty acid transporter. Once in the muscle cell, fatty acids are activated by esterification to CoA by fatty acyl CoA synthetase and can be channelled into storage as intramyocellular TAG or used as substrate for oxidation by mitochondria (Frayn et al., 2006).

1.3. Insulin signalling

1.3.1 The insulin receptor and insulin receptor substrates

Once released by pancreatic β cells into the circulation, insulin exerts its effect on insulin target tissues by binding to the insulin receptor (IR). IR is a membrane glycoprotein consisting of two α and two β subunits organised into an α2β2 heterotetrameric complex and several functional domains. The α-subunit is extracellular and contains the insulin binding site. The binding of insulin to the α-subunit results in a conformational change that activates the intrinsic tyrosine kinase activity of the β-subunit leading to its autophosphorylation on select tyrosine residues (Shoelson et al., 1988). The phosphorylated tyrosine residues act as docking sites for several signalling molecules, which, in turn, bind to downstream insulin-signalling proteins (Kanzaki, 2006). 11 different IR substrates have been identified. Among these

are Grb2-associated binder-1 (Gab1), Src-homology-2-containing protein (SHP-2) and the family of the insulin receptor substrates (IRS) proteins (Taniguchi et al., 2006). IRS proteins represent the first step in the activation of the insulin signalling pathway. The family of IRS proteins comprise six members. (Cai et al., 2003). IRS proteins contain both pleckstrin-homology domains (PH domains) and phosphotyrosine-binding domains (PTB domains) that bind the IR (Taniguchi et al., 2006). IRS2 is structurally different from the others since it is the only one that can bind IR *via* a kinase-regulatory loop binding domain (Sawka-Verhelle et al., 1996). During the transient interaction with the autophosphorylated IR, IRS proteins are phosphorylated on potentially 20 different tyrosine residues, which can then be bound by proteins containing Src-homology domains (SH2 domains). Among the proteins that bind tyrosine-phosphorylated IRS proteins is p85, the regulatory subunit of phosphoinositide-3-kinase (PI3K), a critical node in the insulin signalling network, and the adaptor molecule growth factor receptor-bound protein 2 (Grb2), which mediates the activation of the Ras-MAPK pathway (Virkamaki et al., 1999). In contrast, the SH2-domain-containing tyrosine phosphatase-2 (SHP-2) can bind to IRS1 on two phosphotyrosine residues at its C terminus, and dephosphorylates phosphotyrosines residues that mediate the interaction with PI3K and Grb2. In addition, kinases such as extracellular regulated kinase 1/2 (ERK1/2) and ribosomal protein P70S6 kinase (P70S6K) have been shown to phosphorylate IRS proteins on inhibitory serine residues. Since these kinases are activated by insulin, it follows that serine phosphorylation might act as a negative-feedback mechanism for the insulin pathway (Fritsche et al., 2011). Moreover, inhibitory serine phosphorylation has been identified as one mechanism mediating insulin

resistance. Activation of c-Jun-N-terminal kinase (JNK) by stress and excess free fatty acids results in increased serine phosphorylation of IRS-1 and a consequent reduction in activation of insulin signalling (Aguirre et al., 2000). In addition, tumor necrosis factor α (TNFα), inhibitor of kappa B kinase (IKK) and protein kinase C θ (PKCθ), which are elevated in states of low inflammation (as seen during obesity and diabetes) have been shown to promote insulin resistance through enhanced serine phosphorylation of IRS1 (Draznin, 2006).

1.3.2 Phosphatidylinositide-3-kinase (PI3K)

Phosphoinositide-3-kinase (PI3K) are enzymes that transfer phosphate to position 3 of the phosphoinositide ring, regulating different processes, including the activation of insulin signalling. Three subclasses of PI3K have been identified but only the class I enzymes generate phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) and phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) products *in vivo* (Vanhaesebroeck and Waterfield, 1999). Class IA PI3K exists as an heterodimer comprised of a catalytic subunit of ~110kDa (p110) and a regulatory subunit of ~85kDa (p85) containing an SH2 domain, which is responsible for interaction with p110 (Shepherd et al., 1998). The catalytic subunit is in fact unstable and quickly degraded, therefore it is almost always found in a complex with the regulatory subunit. The interaction between p85 and p110 not only stabilises the catalytic subunit, but also inhibits its enzymatic function. Only when p85 binds to IRS proteins through its SH2-domain, is the inhibition released and p110 is targeted to the plasma membrane where its substrate ($PI(4,5)P_2$) is localised (Klippel et al., 1994). Once $PI(3,4,5)P_3$ is generated at the plasma membrane following insulin stimulation it recruits several PH-domain containing proteins, including the serine/threonine

kinase 3'-phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB/Akt) (Toker and Newton, 2000a, b). As the activation of the IR terminates, $PI(3,4,5)P_3$ is dephosphorylated by the 3' phosphoinositide phosphatase PTEN (phosphatase and tensin homolog) resulting in release of recruited PH-domain containing proteins into the cytoplasm (Maehama and Dixon, 1998).

1.3.3 Protein kinase B (PKB/Akt)

Akt is a serine/threonine kinase that has three isoforms in mammals, Akt 1-3, each encoded by different genes. These isoforms share a common C-terminal catalytic domain and an N-terminal PH domain, which interacts with $PI(3,4)P_2$ and $PI(3,4,5)P_3$ (Coffer et al., 1998). AKT1-3 differ in their biological function and distribution. Akt1 is widely expressed and silencing its expression results in impaired growth but no detectable metabolic abnormalities (Chen et al., 2001). In contrast, Akt2 is chiefly expressed in insulin-target tissues, such as fat and liver (Chan et al., 1999). The finding that Akt2-deficient mice develop insulin resistance suggests a role in modulating insulin-mediated glucose homeostasis and is fully consistent with its expression in these key insulin target tissues (Cho et al., 2001). In contrast, Akt3 plays a role in neuronal development and is predominantly expressed in the nervous system and testis (Tschopp et al., 2005). Upon PI3K activation, Akt is recruited to the plasma membrane by binding $PI(3,4,5)P_3$ *via* its PH domain. This interaction induces a conformational change in Akt which leads to its activation following phosphorylation of two regulatory sites. The first one is a threonine residue, T308, phosphorylated by 3-phosphoinositide-dependent protein kinase-1 (PDK1). Full activation of Akt requires phosphorylation of a regulatory second site, S473, which is catalysed

by mammalian target of rapamycin (mTOR) complex 2 (mTORC2) (Alessi et al., 1996; Sarbassov et al., 2005).

Akt is negatively regulated by numerous proteins. Protein phosphatase-2 (PP2A) and PH-domain leucine-rich repeat protein phosphatase (PHLLP) directly target and dephosphorylate Akt (Brazil et al., 2004; Gao et al., 2005). Tribbles-3 (TRB3) inhibits Akt phosphorylation by binding it (Du et al., 2003) and RNAi-mediated down regulation of its expression results in improved insulin sensitivity *in vivo* (Koo et al., 2004). Additional mechanisms of Akt inhibition, which mediate insulin resistance development, will be discussed later.

1.3.4 Akt-regulated processes

Akt represents a critical node of the insulin signalling pathway, since it acts on downstream targets to regulate several cellular processes including glucose uptake, glycogen synthesis, protein synthesis and cell survival (Fig 1.2) (Manning and Cantley, 2007).

1.3.4.1 Glucose uptake

Insulin stimulates the uptake of glucose into skeletal muscle and adipose tissue through the insulin-responsive GLUT4 transporter, which is expressed exclusively in these tissues. Contrary to other members of the GLUT transporter family, GLUT4 is mainly stored in intracellular vesicles, which translocate from the cytoplasm to the plasma membrane upon insulin stimulation. Once the stimulus is terminated, GLUT4 is internalised by endocytosis and routed to specialised storage vesicles (Bryant et al., 2002; Watson et al., 2004). The molecular mechanism that mediates GLUT4 translocation and consequent facilitated glucose uptake are mediated by Akt. Previous work from the Hundal

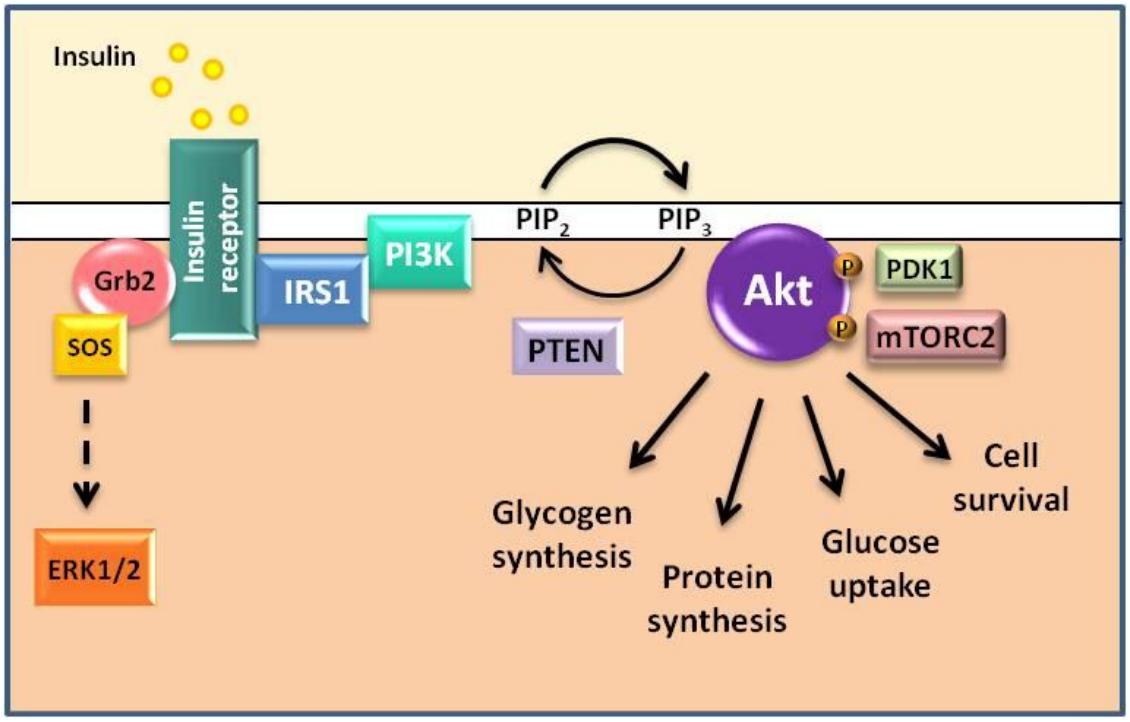


Figure 1.2 Overview of proximal insulin signalling pathway.

Insulin binding to IR triggers phosphorylation of IR and IRS1. IRS1 recruits PI3K, which, once activated, catalyses the phosphorylation of $PI(4,5)P_2$ (PIP_2) to generate $PI(3,4,5)P_3$ (PIP_3). $PI(3,4,5)P_3$ recruits Akt to the membrane. Akt is then activated by phosphorylation on T308 by PDK1 and S473 by mTORC2, and stimulates several downstream pathways. IR activation also results in the binding of Grb2/SOS complex leading to activation of the Ras/Raf/MEK/ERK1/2 pathway.

lab has shown that overexpression of wild-type Akt1 or of a constitutively active membrane-targeted Akt1 results in a significant enhancement in glucose uptake, even in the absence of insulin (Hajduch et al., 1998). In line with this result, over expression of a dominant inhibitory mutant of Akt drastically reduces insulin-induced GLUT4 translocation (Cong et al., 1997). Although the precise mechanism by which Akt regulates glucose uptake remains unclear, it appears to involve its downstream target AS160 (Akt substrate of 160 kDa). AS160 contains six Akt phosphorylation sites and a Rab GTPase activating protein (GAP) domain (Miinea et al., 2005). The phosphorylation of AS160 by Akt in response to insulin reduces its Rab GAP activity, which allows a Rab-family GTPase associated to GLUT4 vesicles to become guanosine triphosphate (GTP) loaded and stimulate vesicle translocation (Sakamoto and Holman, 2008; Sano et al., 2007).

1.3.4.2 Glycogen synthesis

One of the main processes regulated by insulin is the storage of glucose as glycogen. Glycogen is synthesised from uridine diphosphate (UDP)-glucose monomers by glycogen synthase (GS) (Lawrence and Roach, 1997). Two main mechanisms by which insulin regulates GS activity have been indentified- one involving allosteric regulation by glucose-6-phosphate (G6P) (Bouskila et al., 2010) and the other covalent phosphorylation (Friedman and Larner, 1963). The latter can be accomplished by glycogen synthase kinase 3 α/β (GSK3α/β), a serine/threonine kinase responsible for the phosphorylation of several serine residues on GS and its consequent inhibition. Once activated by insulin, Akt phosphorylates GSK3α on S21 and GSK3β on S9 promoting inactivation of the

kinase and therefore alleviating its inhibition of GS (McManus et al., 2005; Sutherland et al., 1993).

1.3.4.3 Cell growth and protein synthesis

Upon insulin stimulation, Akt promotes protein synthesis through the activation of the mTOR complex 1 (mTORC1)/ P70S6K pathway. mTORC1 signalling plays a critical role in the regulation of mRNA translation and ribosome biogenesis, and can be activated not only by insulin but also by growth factors and nutrient provision (Wullschleger et al., 2006). Akt-mediated activation involves phosphorylation and consequent inactivation of the tuberous sclerosis complex 2 (TSC2), a negative regulator of mTORC1. TSC2 is found in a complex with TSC1 and acts as a GAP protein for Rheb. This is a Ras-related small G protein, which activates mTORC1 when it is in an active GTP-loaded state. Therefore, inhibition of TSC2 promoted by Akt phosphorylation of S939 and T1462 residues allows Rheb-GTP to activate the mTORC1 pathway (Inoki et al., 2002; Manning et al., 2002). mTORC1 signalling and the molecular mechanisms mediating mRNA translation will be discussed in detail in section 1.11.1.

1.3.4.4 Cell survival

Numerous studies have reported the ability of Akt to promote the survival of cells, principally by inhibiting proteins with pro-apoptotic function. The enhanced cell survival is in fact due to negative regulation of expression or function of Bcl-2 homology domain (BH3)-only proteins, which induce apoptosis by binding and inhibiting prosurvival B-cell lymphoma 2 (Bcl-2) family proteins. For example, Datta *et al.* have shown that Akt can directly phosphorylate the BH3-only protein

Bcl-2-associated death promoter (BAD) on S316. This event creates a docking site, which can be bound by 14-3-3 proteins leading to the release of BAD target proteins B-cell lymphoma-extra large (BCL-X_L) (Datta et al., 1997; Datta et al., 2000). Akt can also exert its prosurvival effect by negative regulation of transcription factors like p53 and Forkhead box-O proteins (FoxOs). The latter ones regulate the expression of pro-apoptotic members of the Bcl2-family, such as BIM, cyclin-dependent kinase inhibitors (CDKIs) and death receptor ligands such as FAS ligand and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Brunet et al., 1999; Kikuchi et al., 2007). In the nucleus, Akt directly phosphorylates FoxO proteins on T24 and S256 thereby creating a binding site for 14-3-3 proteins, which sequester FoxOs displacing them from target genes (Zhang et al., 2011). Cell survival is also promoted by induction of the nuclear factor kappa B (NF-kB) pathway as a result of direct phosphorylation and consequent activation of IκB kinase α (IKKα). Once activated, IKKα phosphorylates and targets for proteasomal degradation inhibitor of kappa B α (IKBα), a negative regulator of NF-kB. The dissociation and loss of IKBα then allows NF-kB to translocate to the nucleus and promote the transcription of prosurvival genes (Dan et al., 2008).

1.4 Mitogen activated protein kinase (MAPK) signalling pathway

In addition to the PI3K/Akt axis, insulin stimulation leads to the activation of the MAPK-ERK1/2 pathway (Ray and Sturgill, 1987). ERK1/2 belongs to one of the serine/threonine MAPK families; other members of this family include JNK and p38 MAPK. All MAPK pathways contain a three-tiered kinase cascade consisting of an upstream MAPKKK, which phoshporylates a MAPKK, which activates the MAPK enzyme by dual phosphorylation on threonine and tyrosine

residues within the motif T-Xaa-Y (Johnson and Lapadat, 2002). While JNK and p38 are strongly activated by cytokines and stress stimuli, ERK1/2 activation is mainly induced by growth factors. The MAPK ERK1/2 pathway is activated by insulin following the recruitment of the Grb2-SOS (Son of sevenless protein) complex, which leads to the activation of Ras, Raf and MEK1/2 (mitogen-activated extracellular-regulated kinase 1/2) (Diaz et al., 1997; Johnson and Lapadat, 2002; Marais et al., 1995; Skolnik et al., 1993). Once phosphorylated MEK1/2 can activate their sole substrates ERK1/2 by phosphorylation on T202 and Y204 in the activation loop (Kolch, 2000; Lewis et al., 1998). ERK1/2 have a wide range of substrates, including growth factor-responsive targets in the cytosol but also several transcription factors regulating gene expression in the nucleus. The major target of ERK1/2 is 90 kDa ribosomal protein S6 Kinase (p90RSK). Once activated, p90RSK migrates to the nucleus where it activates several transcription factors including, for example, the product of the proto-oncogene c-Fos at S362, SRF (Serum Response Factor) at S103, and CREB (Cyclic AMP Response Element-Binding protein) at S133 (Dalby et al., 1998), involved in the regulation of cell proliferation, differentiation and survival (Persengiev and Green, 2003). ERK1/2 can also migrate to the nucleus and directly phosphorylate transcription factors, such as Elk1 (Kortenjann et al., 1994). In insulin signalling the ERK1/2 MAPK pathway is associated principally with promotion of cell proliferation and differentiation and does not have a critical role in the regulation of metabolic effects induced by the hormone (Denton and Tavare, 1995). Since MAPK activation requires dual phosphorylation, it follows that their activity is negatively regulated by phosphatases. Indeed, the serine/threonine phosphatase PP2A has been

identified as a negative regulator of the MEK1/2/ERK1/2 pathway (Alessi et al., 1995). Another important mediator of this regulation is a family of 10 dual-specificity MAPK phosphatases (DUSPs), which are able to dephosphorylate both threonine and tyrosine residues on MAPK (Owens and Keyse, 2007)

1.5 Fatty acid metabolism

1.5.1 Fatty acid synthesis

Fatty acid synthesis is a multi-step process occurring in the cytosol and regulated by the fatty acid synthase (FAS), a complex multifunctional enzyme of ~250 kDa (Jayakumar et al., 1995). The FAS enzyme coordinates the synthesis of palmitate, the most prevalent saturated fatty acid, from acetyl CoA, malonyl CoA and NADPH by the repetition of the following reaction sequence: condensation, reduction, dehydration and reduction (Fig 1.3). All the intermediates of these reactions are linked to an acyl carrier protein (ACP) (Chirala and Wakil, 2004). Palmitate is the only fatty acid produced by FAS, but longer fatty acids can be produced by a membrane-bound enzyme known as elongation of very-long-chain fatty acids (Elovls) (Jakobsson et al., 2006). The elongation process consists of the addition of two-carbon units, provided by malonyl CoA, to the carboxyl end of the fatty acyl CoA substrate. This occurs in four separate enzymatic reactions, which follow those involved in fatty acid synthesis. The elongase enzymes catalyse the initial and rate-controlling condensation reaction (Guillou et al., 2010). A combination of elongation and desaturation brings about the production of unsaturated fatty acids. The desaturation reaction is catalysed by acyl-CoA desaturase, an enzyme that inserts a double bond at a specific position in the fatty acid carbon chain. Three

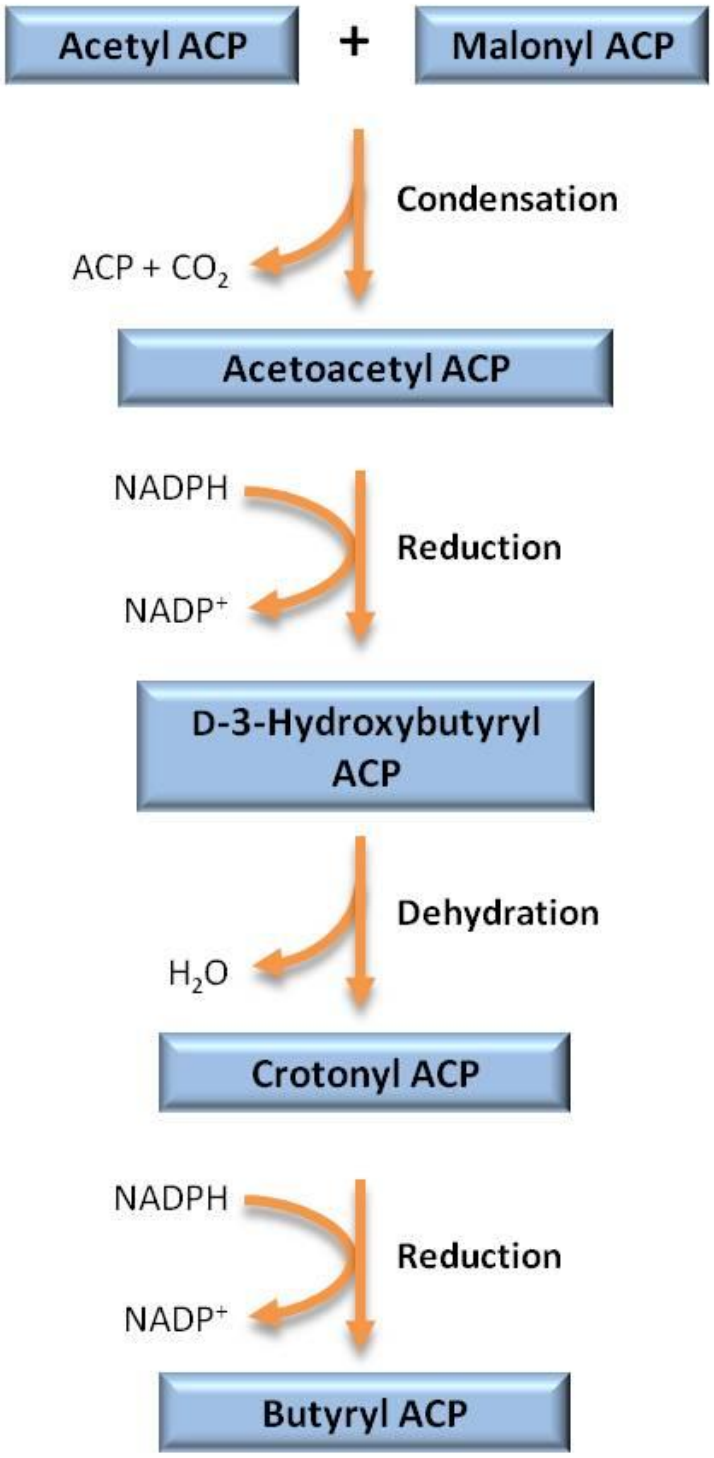


Figure 1.3 Fatty acids synthesis.

Fatty acids are synthesised by the repetition of the following reaction sequence: condensation, reduction, dehydration and reduction.

desaturases are present in mammals, Δ5, Δ6 and Δ9 desaturase, which can insert a double bond at carbon 5, 6 and 9 respectively. The desaturases can be divided into two families: fatty acid desaturases (FADs) and stearoyl-CoA desaturases (SCDs). Δ6 and Δ5 desaturases are FADs and are involved in the synthesis of highly unsaturated fatty acids, which are mainly esterified into phospholipids, while Δ9 is a SCD and is required for the generation of mono-unsaturated fatty acids, like oleic acid (Nakamura and Nara, 2004; Paton and Ntambi, 2009). Mammals do not have the enzyme to introduce a double bond after C9 of a fatty acid chain and consequently fatty acids such as linoleic acid (C18:2, n-9,12) and linolenic acid (C18:3, n-9,12,15), known as essential FAs, have to be acquired by diet (Plourde and Cunnane, 2007). The process of fatty acid synthesis is activated when carbohydrate availability is high and inhibited during starvation, when fatty acids are released by adipose tissue by the action of lipases. One of the key proteins involved in the tight regulation of this process is acetyl CoA carboxylase (ACC) (Fig 1.4). ACC catalyses the formation of malonyl-CoA from acetyl-CoA, the first step in fatty acid synthesis, and can be regulated in two ways: allosterically by citrate and through reciprocal phosphorylation. When glucose levels are high, glucose-derived acetyl-CoA can be utilised for synthesis of fatty acids. In order to be transferred from mitochondria to the cytosol, where fatty acid synthesis occurs, it has to be converted into citrate (Berg 2002). Citrate levels are high when adequate acetyl-CoA enters into the Krebs cycle, and allosterically activates ACC in order to convert excess acetyl-CoA into malonyl-CoA (Ruderman et al., 1999). Increasing concentrations of malonyl-CoA inhibit carnitine palmitoyl-transferase-1 (CPT-1), which mediates the entry of fatty acids into the mitochondria to

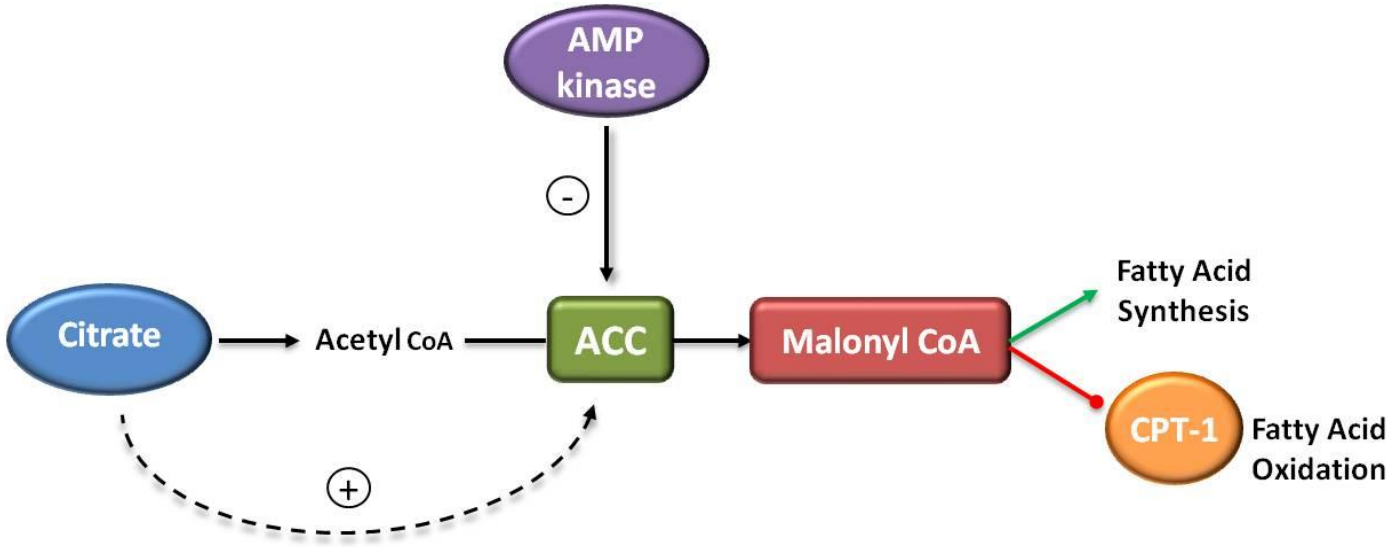


Figure 1.4 ACC-mediated regulation of fatty acid synthesis.

High-glucose availability results in increased levels of citrate, which allosterically activates ACC to convert acetyl-CoA into malonyl-CoA. Increasing concentration of malonyl-CoA leads to synthesis of fatty acids, while inhibiting CPT-1 and fatty acid oxidation. Negative regulation of ACC is regulated by its upstream kinase AMPK, which inactivates it by phospohrylation on S79. Modified from (Ruderman et al., 1999).

promote their oxidation (Chien et al., 2000; Ruderman et al., 1999). ACC activation promoted by citrate can be antagonised by palmitoyl-CoA, which is abundant when fatty acid levels are high and additional fatty acid synthesis is not required (Ogiwara et al., 1978). As mentioned above, acetyl CoA can also be regulated by phosphorylation. Under low glucose conditions a decreased adenosine triphosphate (ATP)/adenosine diphosphate (ADP) ratio will prevail and this will, in turn, result in the activation of adenosine monophosphate (AMP)-activated protein kinase (AMPK). The latter serves as the upstream kinase of ACC. AMPK phosphorylates and inactivates ACC on S79 thereby preventing the synthesis of malonyl-CoA and promoting fatty acid oxidation instead (Hardie and Pan, 2002).

1.5.2 Fatty acid uptake

Entry of fatty acids into the cells was thought to occur primarily by passive diffusion across the membrane, with the rate of uptake determined by the fatty acid concentration in the blood circulation and the rate of intracellular fatty acid metabolism. The aqueous solubility of fatty acids is recognised as being extremely low therefore fatty acids need to be bound to protein for vascular transport (Vorum et al., 1992). Albumin and fatty acid binding protein (FABPc) act as buffers, within the extracellular and intracellular compartment respectively. Therefore under physiological conditions the concentration of fatty acids is 100-400 μM, but only 7.5 ± 2.5 nM is not bound to proteins (Richieri and Kleinfeld, 1995). The uptake of fatty acids into cells can occur by passive diffusion or be mediated by membrane proteins (Kampf and Kleinfeld, 2004). The most common membrane associated fatty acids transporters are shown in Table 1.1 (Glatz et al., 2010).

Protein	**Molecular weight (kDa)**	**Sites of expression**
$FABP_{pm}$ (Plasma membrane fatty acid binding protein)	40-43	Liver, heart, muscle, adipose tissue, intestine, placenta
FATP1 (Fatty acid transport protein 1)	63	Adipose tissue, heart, muscle brain, kidney, skin, lung
FAT (Fatty acid translocase)/CD36	88	Heart, intestine, skeletal muscle, adipose tissue, spleen, platelets, monocyte/macrophage, endothelium, epidermis, kidney, brain, liver
Caveolin 1	21-24	Ubiquitously expressed, except in muscle and heart where caveolin 3 is predominantly expressed

Table 1.1 Tissue expression and molecular weight of most common plasma membrane proteins that mediate fatty acid uptake.

Modified from (Glatz et al., 2010)

1.5.3 Fatty acid oxidation

Kennedy and Lehninger demonstrated in 1949 that fatty acids were oxidised in mitochondria (Kennedy and Lehninger, 1949). Before entering into this organelle fatty acids need to be activated to fatty acyl-CoA. This process involves esterification of fatty acids with acyl-CoA and is mediated by acyl-CoA synthetase on the outer mitochondrial membrane. Once activated, fatty acyl-CoAs have to reach the mitochondrial matrix, where the oxidation process occurs. Long-chain fatty acyl-CoAs (LCFA-CoA) can not easily traverse the inner mitochondrial membrane, therefore they need to be conjugated to carnitine, a reaction catalysed by carnitine palmitoyl transferase (CPT) 1. CPT-1 is located on the cytosolic face of the external mitochondrial membrane and represents the rate-limiting enzyme for fatty acid oxidation. Carnitine acylcarnitine translocase (CACT) mediates the transfer across the inner mitochondrial membrane of LCFA-carnitine, which is cleaved by CPT-2. Carnitine is released into the matrix and can be recycled back to the cytosol through CACT, while long-chain fatty acids are conjugated back to CoA form (Longo et al., 2006) (Fig 1.5). Fatty acyl-CoA can then be degraded to acetyl-CoA *via* the β-oxidation process, a cycle of four reactions in which fatty acyl-CoAs are shortened by removal of two carboxy terminal carbon atoms that are released as acetyl-CoA (Fig 1.6). This cycle is repeated until all the acyl-CoA is converted into acetyl-CoA. The acetyl-CoA can then enter into the tricarboxylic acid cycle to produce ATP *via* the respiratory chain and ATP-synthase. (Beinert et al., 1956; Houten and Wanders, 2010). β-oxidation of monounsaturated and polyunsaturated fatty acids share the same reactions as for saturated fatty

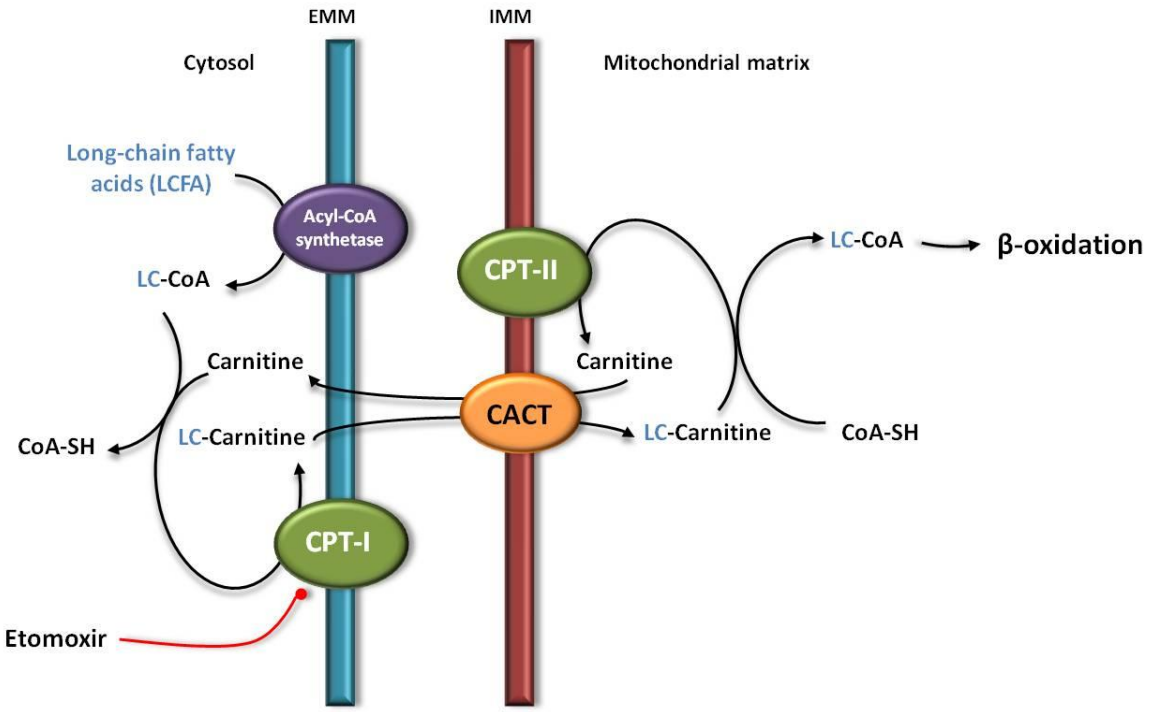


Figure 1.5 Transport of long chain fatty acids into the mitochondria.

Long chain fatty acids (LC-FA) are converted in the cytosol to LCFA-CoA, a reaction catalysed by the enzyme long-chain fatty acyl CoA synthetase, localised on the external mitochondrial membrane (EMM). To be transported across the EMM, LC-CoA are converted to LC-carntine by carnitine palmitoyltransferase I (CPT-1) in a reaction that regenerates free CoA. Carnitine acylcarnitine translocase (CACT) regulates the transfer of LC-carnitine across the inner mitochondrial membrane (IMM). Once in the mitochondrial matrix, CPT-2 catalyses the conversion of LC-carnitine to LC-CoA, which can now be oxidised. This process generates free carnitine that can be redirected to the cytosol throught CACT. Fatty acid oxidation can be blocked by using the CPT-1 inhibitor Etomoxir.

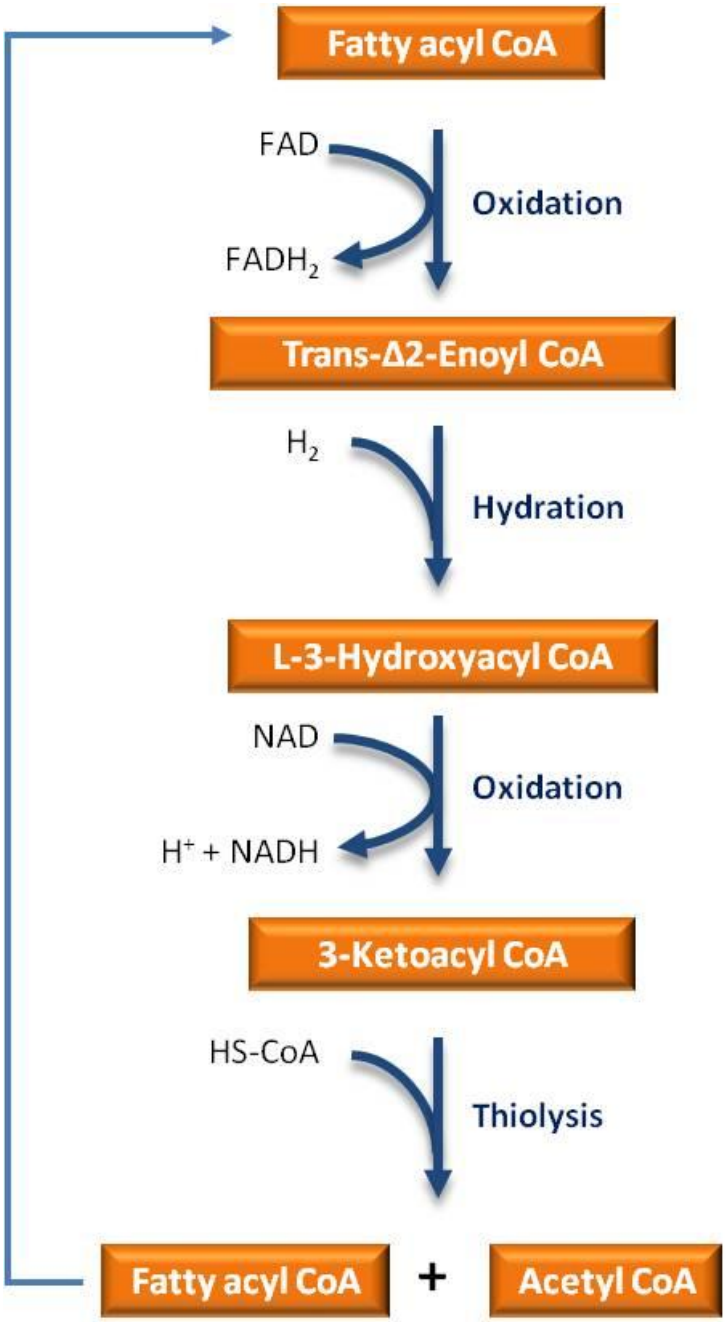


Figure 1.6 Fatty acid oxidation.

Fatty-acyl CoA are oxidised in a four step process in which the fatty acyl cabon chain is sequentially shortened by two carbons that are released as acetyl CoA. The process is repeated until the fatty acyl CoA is completely oxidised.

acids, but require two additional enzymes, isomerase and epimerase respectively. These enzymes shift the configuration of certain reaction intermediates to produce regular substrates for the subsequent reaction (Berg, 2002; Hiltunen and Qin, 2000).

Fatty acid oxidation is a tightly regulated process. In fact, organs can select which substrate to use to maintain energy homeostasis and therefore switch off glucose or fatty acid oxidation according to fuel availability. There are several mechanisms for the regulation of cellular fuel use. As mentioned in section 1.5.1, when carbohydrates are in excess, citrate accumulation derived from glucose oxidation leads to activation of ACC and consequent production of malonyl CoA. Malonyl CoA inhibits CPT-1, the rate-limiting enzyme of fatty acid oxidation, and serves as a substrate for fatty acid synthesis. Moreover when blood glucose is high, the increased delivery and utilisation of glucose promotes inhibition of lipolysis thus suppressing the availability of fatty acids as a competing fuel substrate (Hue and Taegtmeyer, 2009; Ruderman et al., 1999). In contrast, in the fasting state, when circulating glucose is low fatty acids become the main energy source. Under such circumstances, glucagon stimulates HSL, inducing lipolysis and release of free fatty acids from adipose tissue. This event is significant as fatty acids can bind and activate peroxisome proliferator-activated receptor (PPARs), such as PPARα, which stimulate fatty acid utilisation by activating pathways regulating their transport, esterification and oxidation (Mandard et al., 2004). Moreover, during starvation or glucose deprivation, the ratio of AMP/ATP rises resulting in the activation of AMPK and consequent inhibition of its downstream target ACC. ACC inhibition leads to CPT-1 activation, inducing energy production by fatty acid oxidation. In addition,

fatty acid oxidation impairs glucose metabolism by short-term inhibition of different glycolytic steps. For example, high level of β-oxidation increases mitochondrial ratio of acetyl CoA/CoA, which activates the pyruvate dehydrogenase kinase (PDHK). Pyruvate dehydrogenase catalyses the oxidative decarboxylation of pyruvate to acetyl-CoA thus linking glycolysis to oxidative metabolism and can be inhibited by PDHK-mediated phosphorylation. The latter results in cytosolic citrate accumulation, which has been demonstrated to inhibit other glycolytic enzymes such as phosphofructokinase. This reciprocal competition between the oxidation of glucose or fatty acids is known as the “Randle cycle” (Garland et al., 1963; Randle et al., 1963).

1.5.4 TAG synthesis

In the well-fed state, glucose serves as the primary source of energy, so fatty acids are channeled into TAG synthesis. TAG represent highly concentrated stores of energy as they are much more reduced than carbohydrates and do not bind water. The complete oxidation of fatty acids produces 9 kCal/g against the 4 kCal/g of carbohydrates, explaining why the body has selected TAG over glycogen as the major energy store. Although they can be synthesised in almost every cell, adipocytes are higly specialised for synthesis and storage of TAG (occupying ~90% of cell volume) and for their mobilization during the fasting state (Berg, 2002). There are three different pathways involved in the synthesis of TAG: the glycerol-phosphatase (GP) pathway in mitochondria or endoplasmatic reticulum (ER), the dihydroxyacetone phosphate pathway in peroxisomes and the monoacyglycerol (MAG) pathway in the ER (Coleman and Mashek, 2011; Kindel et al., 2010). In most mammalian cells the major pathways involve GP and MAG (Coleman and Mashek, 2011) and their

reactions are shown in Fig 1.7. During fasting stored TAG is catabolised by lipolysis, a process that releases fatty acids from the glycerol backbone to be metabolised or released into the blood circulation for use in other tissues. This process occurs in every cell, but mainly in adipocytes given their specialised role in the storage of TAG. Although initially HSL was thought to be the only enzyme involved in TAG hydrolysis, it is now clear that the major TAG hydrolase in adipose tissue is the adipose TAG Lipase (ATGL) (Zimmermann et al., 2009; Zimmermann et al., 2004). Both ATGL and HSL can catalyse the hydrolysis of TAG to produce diacylglycerol (DAG) (Zimmermann et al., 2004). DAG is then preferentially hydrolysed by HSL (Fredrikson et al., 1981; Haemmerle et al., 2002), while ATGL presents a really weak affinity for this substrate (Zimmermann et al., 2004). To date, HSL is recognised as the main lipase responsible for breaking down of both DAG and MAG and preferably hydrolyses *sn*-1(3) ester bonds (Yeaman, 2004). Therefore, an additional 2-ester monoacylglycerol lipase is required in adipocytes to act with HSL to ensure a complete breakdown of TAG (Fredrikson et al., 1986). Lipolysis plays an important role not only in the release and redistribution of fat as fuel, but also in the production of lipid molecules that are involved in several signalling pathways. Products of TAG catabolism, such as DAG, ceramide and fatty acids, are in fact involved in the modulation of cellular pathways like insulin signalling or regulation of gene transcription (Zechner et al., 2012).

1.6 Saturated fatty acids (SFAs) and their effect on insulin signalling

There is considerable evidence that sustained increase in the blood levels of SFAs result in the development of insulin resistance. Insulin resistance is described as the reduction in insulin ability to stimulate glucose uptake and

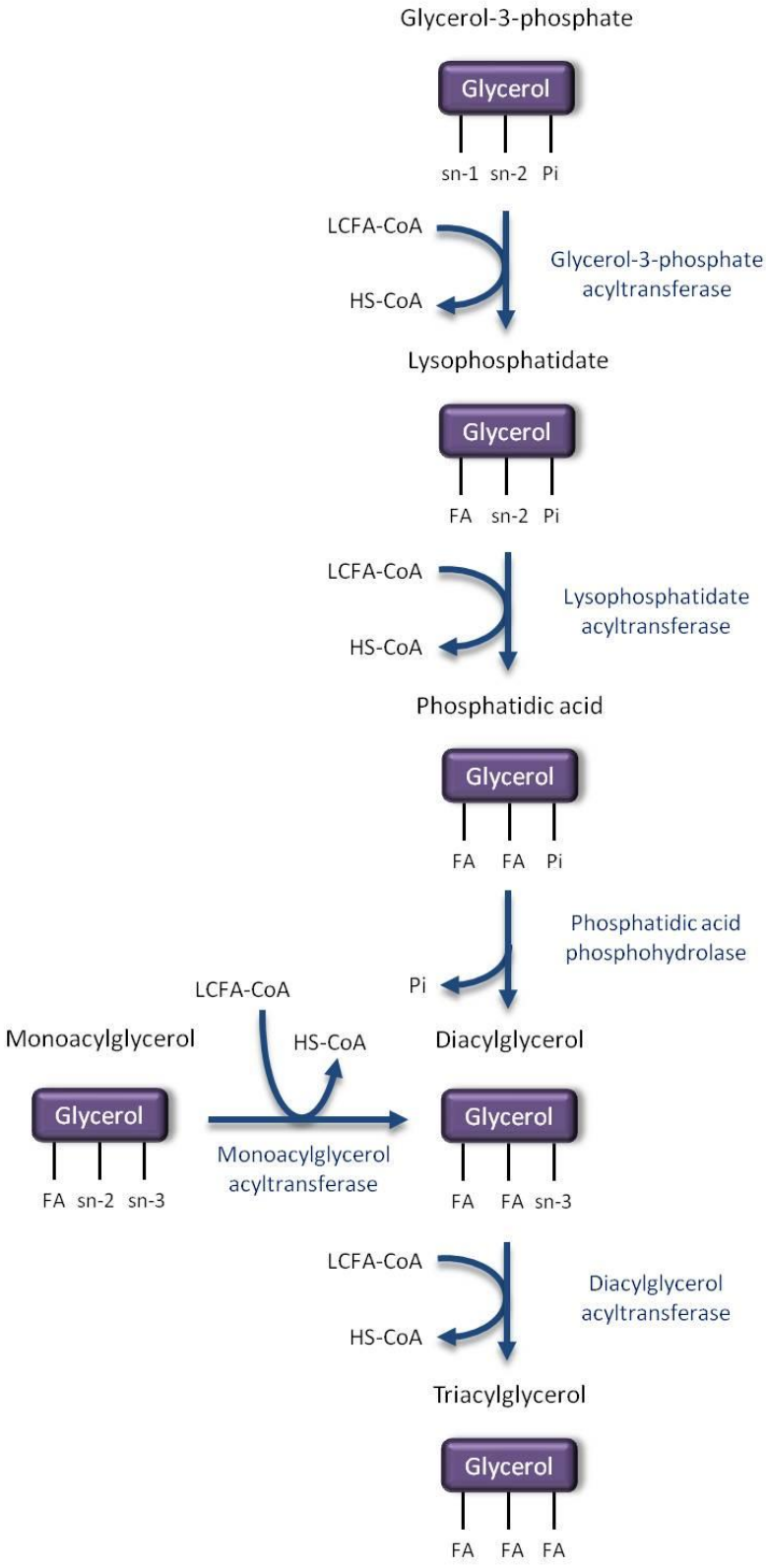


Figure 1.7 Glycerol phosphate and monoacylglycerol pathways.

Long-chain fatty acyl CoA (LCFA-CoA) are sequentially esterified to a glycerol backbone to generate diacylglycerol and triacylglycerol.

other metabolic processes in insulin-target tissues (Martins et al., 2012). Since insulin-induced glucose uptake occurs mainly in skeletal muscle, it follows that free SFAs may cause insulin resistance by primarily exerting their effect in this tissue. Indeed, chronic exposure of myotubes to SFAs, such as palmitate, results in a significant impairment of insulin signalling (Boden, 2006; Dimopoulos et al., 2006; Feng et al., 2012; Yuzefovych et al., 2010). Although the exact molecular mechanisms by which they exert this negative effect is unclear, it is recognised that accumulation of lipid derivatives (Macrae et al., 2013; Stratford et al., 2004; Watson et al., 2009), oxidative stress (Hirabara et al., 2010; Lambertucci et al., 2008), mitochondrial dysfunction (Dela and Helge, 2013; Hirabara et al., 2010; Yuzefovych et al., 2010) and inflammation (Boden, 2006; Green et al., 2011; Salvado et al., 2013) induced by SFAs may be contributing factors (Fig 1.9).

1.6.1 DAG and PKC

DAG is one of the candidate molecules implicated in the development of insulin resistance by SFAs. In high-fat fed rats a two-fold increase in DAG levels has been found to be associated with the development of tissue insulin resistance (Schmitz-Peiffer et al., 1997). DAG can be synthesised *de novo via* GP or MAG pathways (Coleman and Mashek, 2011; Timmers et al., 2008), or by hydrolysis of TAG by HSL. Furthermore, it is noteworthy that DAG is not only an intermediate of TAG metabolism, but also plays a role in regulating intracellular signalling (Schmitz-Peiffer, 2000). Indeed, DAG is a potent activator of conventional and novel protein kinase C (PKC) isoforms, which have been implicated in the pathogenesis of insulin resistance (Heydrick et al., 1991; Schmitz-Peiffer et al., 1997). PKC is a family of serine/threonine kinases that

can be activated by transient increase in intracellular DAG in a Ca^{2+} dependent manner or in response to tumor-promoting phorbol esters. Based on structure, substrate specificity and cofactor requirement PKC kinases are classified into three groups: conventional (α, βI, βII and γ), novel (δ, ε, θ and η) and atypical (ζ and ι/λ) (Newton, 1995; Timmers et al., 2008). Numerous studies have linked the activation of PKC with insulin resistance, especially if that is mediated by increased lipid availability (Heydrick et al., 1991; Itani et al., 2002; Schmitz-Peiffer et al., 1997). In particular, elevated levels of DAG lead to the activation of the classical PKCs α and βI, and of the novel PKCs ε, δ and θ, which negatively regulate insulin signalling mainly *via* inhibitory phosphorylation of IRS1 on its serine residues (Samuel et al., 2010). Indeed, once activated PKCθ can inhibit IRS1 by increasing its phosphorylation on S301, 302, 307 and 1101 (Li et al., 2004; Samuel et al., 2010; Werner et al., 2004). PKCε has been shown to impair insulin signalling by phosphorylation on S636/639 (Mack et al., 2008) and by direct association with the IR, which results in its decreased activity (Ikeda et al., 2001). In addition, in skeletal muscle PKCδ promotes phosphorylation of IRS1 on S357, which is associated with an attendant reduction in insulin action (Waraich et al., 2008).

1.6.2 Ceramide

Ceramide is an important lipid intermediate, which links inflammatory cytokines, such as TNFα, and SFAs to the regulation of insulin signalling. It is a sphingolipid that can be generated by *de novo* synthesis or sphingomyelin hydrolysis. *De novo* synthesis of ceramide can be induced by stress stimuli (*e.g.* UV radiation) and cytokines (*e.g.* TNFα) and starts from the condensation of palmitoyl-CoA and serine; a rate-limiting reaction catalysed by serine

palmitoyltransferase (SPT) (Fig 1.8). Since this enzyme has great affinity for long chain SFAs, it follows that increased levels of ceramide are linked to lipid oversupply and insulin resistance (Summers, 2006). Indeed, chronic exposure of adipocytes and myotubes to palmitate (C16:0), stearate (C18:0), arachidate (C20:0) and lignocerate (C24:0) but not unsaturated fatty acids induces ceramide synthesis (Chavez and Summers, 2003). Studies performed in rodents with streptozotocin-induced diabetes show a 78% increase in intramuscular ceramide levels (Gorska et al., 2004), while in muscle of obese human subjects ceramide content was doubled compared to the insulin-responsive subjects (Straczkowski et al., 2004). The notion that ceramide may contribute to fatty acid induced-insulin resistance is supported by the finding that treating myotubes with an SPT inhibitor protects them from the detrimental effects of palmitate on insulin signalling (Powell et al., 2004; Watson et al., 2009). Several groups have shown that ceramide impairs insulin signalling by suppressing activation of Akt by two distinct, but not mutualy exclusive mechanisms (Chavez et al., 2003; Powell et al., 2003; Stratford et al., 2001; Stratford et al., 2004). First, ceramide inhibits Akt activation by preventing its translocation to the plasma membrane. Previous work from the Hundal group has shown that ceramide can activate atypical PKCζ, and that this results in increased association of Akt with PKCζ and phosphorylation of the former by the latter on T34 within the Akt PH domain. This event results in decreased affinity of Akt for the phosphoinositide PI(3,4,5)P_3. Using a T34A mutated AKT or expressing a dominant-interfering kinase dead-PKCζ mutant ameliorated the

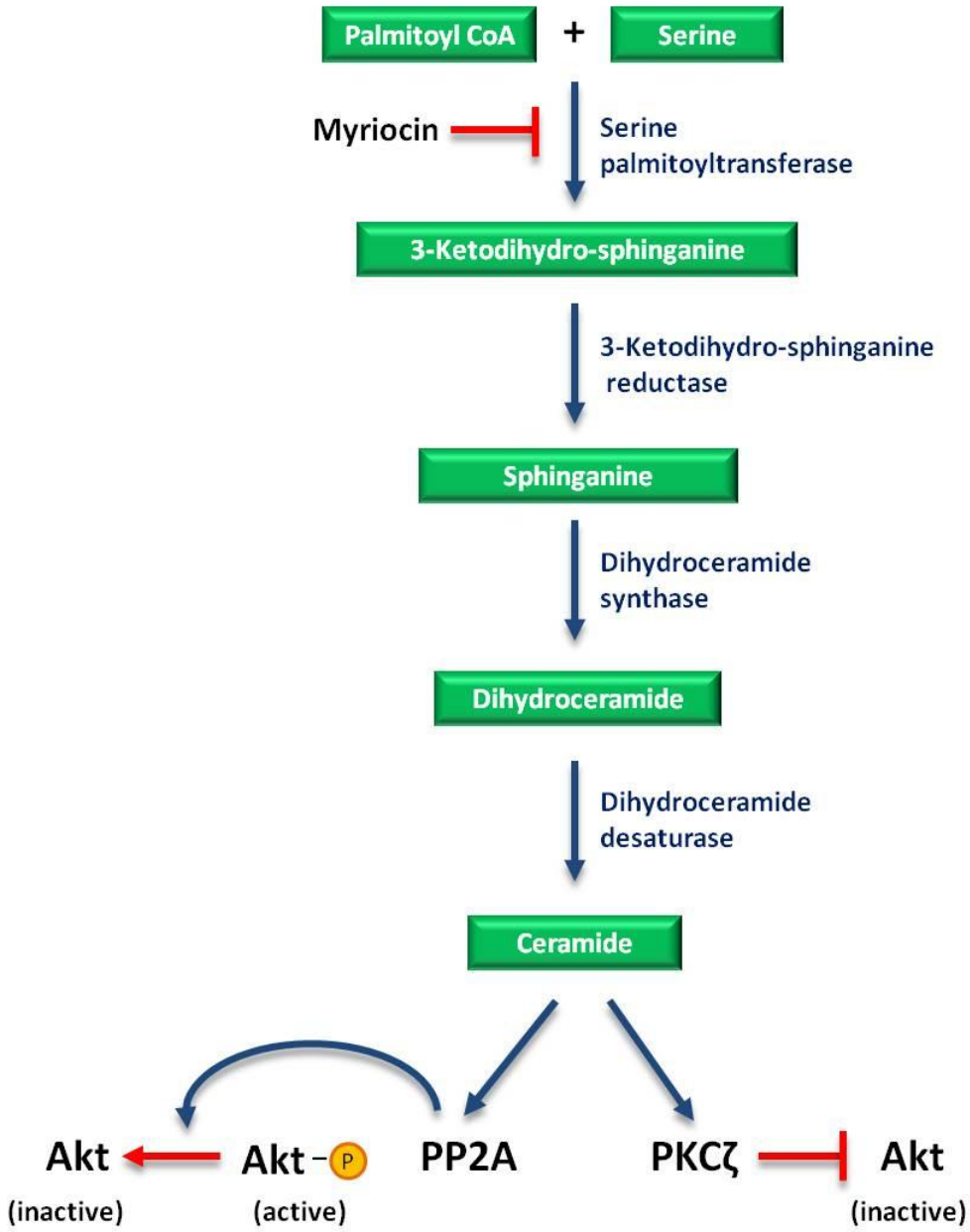


Figure 1.8 *De novo* ceramide synthesis pathway.

Ceramide is synthesised in a 4 step process that is initiated with the condensation of palmitoyl-CoA and serine. This rate-limiting reaction of ceramide synthesis is catalysed by serine palmitoyltransferase, an enzyme that can be inhibited by myriocin. Ceramide can impair insulin signalling by the activation of phosphatase PP2A and kinase PKCζ.

negative effects of ceramide on Akt-PI(3,4,5)P_3 interaction and its insulin-mediated activation (Powell et al., 2003). It has also been noticed that ceramide induces recruitment of Akt and PKCζ to caveolin-enriched microdomains, where their interaction with each other is stabilised and inhibition of Akt is further enhanced (Hajduch et al., 2008). Second, ceramide has been shown to activate PP2A, a phosphatase responsible for the dephosphorylation and consequent inhibition of Akt (Dobrowsky et al., 1993). Treatment with okadaic acid (Chavez et al., 2003; Salinas et al., 2000) or overexpression of the SV40 (simian virus 40) small T antigen (Yang et al., 1991) both inhibit PP2A and, as such, these negate the inhibitory effect of ceramide on Akt activation. Ceramide also appears to induce insulin resistance by promoting ER stress (Boslem et al., 2011), mitochondrial stress (Bikman and Summers, 2011) and inducing pro-inflammatory signalling (Grigsby and Dobrowsky, 2001; Holland et al., 2011).

1.6.3 TAG

Evidence in literature shows that increased fatty acid availability, as seen during obesity, causes a loss in skeletal muscle insulin sensitivity and that this is associated with increased intramyocellular triacylglycerol (TAG) deposition (Forouhi et al., 1999). However, there is mounting evidence to suggest that accumulation of fatty acids as neutral lipid may in fact serve as a protective mechanism that helps counter the effect of lipotoxic intermediates that would otherwise build up in muscle. This proposition is supported by rodent studies showing that high-fat feeding in mice over-expressing diacylglycerol acyltransferase 1 (DGAT1), which facilitates TAG synthesis, protects mice from developing insulin resistance (Timmers et al., 2011).

1.6.4 Pro-inflammatory signalling

There is mounting evidence that activation of the NFkB pathway and increased level of pro-inflammatory cytokines are involved in the development of obesity-induced insulin resistance. Indeed, SFAs can activate inflammatory pathways in different cell types by both direct interaction with Toll-like receptors (TLRs) and indirect modulation of cytokine release, such as that of TNFα, interleukin (IL)-6, IL-8 (Haversen et al., 2009) and IL-1β (Green et al., 2011; Joshi-Barve et al., 2007). SFA-induced TLR activation in turn promotes activation of JNK and IKK, which induces NFkB activation and inhibition of insulin signalling *via* phophorylation of IRS1 on inhibitory serine residues (Aguirre et al., 2000; Huang et al., 2012; Solinas and Karin, 2010). This is further supported by the finding that mice with TLR-4 loss of function mutation are protected from fat-induced inflammation (Tsukumo et al., 2007) and obese mice treated with a JNK-inhibitory peptide exhibit improved insulin sensitivity and glucose tolerance (Kaneto et al., 2004). Holland *et al.* have shown that fatty acid-driven synthesis of ceramide is initiated by TLR-4 receptor and IKK activation since over-expression of a dominant negative-IKK mutant in skeletal muscle cells antagonised palmitate-induced ceramide synthesis and insulin resistance (Holland et al., 2011). In insulin target tissues, TLR activation by SFAs induces secretion of pro-inflammatory cytokines, such as TNFα and IL-6, which can act on peripheral tissues leading to impair insulin signalling (Cai et al., 2005; Jove et al., 2006). TNF-α expression is elevated in both obese rodents and humans (Hotamisligil et al., 1993; Kern et al., 2001) and its inhibition using a TNF-α-neutralising antibody leads to improved insulin sensitivity in obese rats (Hotamisligil et al., 1993). TNF-α can transiently activate the JNK and IKK

inflammatory pathways leading to negative modulation of insulin signalling by serine phosphorylation and inhibition of IRS1 (Liu et al., 1996). Moreover, TNF-α infusion in healthy human subjects has been shown to induce insulin resistance in skeletal muscle by impairing Akt-mediated phosphorylation of AS160, thus leading to decreased whole-body glucose uptake (Plomgaard et al., 2005). Like TNF-α, IL-6 levels are also increased in obese subjects (Kern et al., 2001). IL-6 can be secreted by different cell types, including adipocytes, macrophages and skeletal muscle where it induces insulin resistance *via* impaired IRS-1/PI3K association and increased acyl-CoA levels (Kim et al., 2004).

1.6.5 Mitochondrial dysfunction and oxidative stress

There is increasing evidence that mitochondrial dysfunction and oxidative capacity have a primary role in the pathophysiology of insulin resistance. Indeed, a significant reduction in mitochondrial function and fatty acid oxidation has been observed in skeletal muscle of insulin resistant obese and type 2 diabetic patients (Hulver et al., 2003; Kim et al., 2000). Several studies suggest an important role for SFAs in the induction of mitochondrial dysfunction associated with insulin resistance. High-fat diets in both humans and rodents result in reduced ATP synthesis and oxidative phosphorylation (Brehm et al., 2006; Sparks et al., 2005) while chronic exposure of cultured skeletal muscle cells to palmitate induces increased reactive oxigen species (ROS) production and impairs fatty acid oxidation (Pimenta et al., 2008; Yuzefovych et al., 2010). One main mechanism by which fatty acids can exert this negative effect on mitochondria is by altering expression of genes involved in mitochondrial function and biogenesis. Studies performed both in mice and humans have

shown that a high-fat diet induces a significant decrease in expression of peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1alpha); a master regulator of genes crucial for oxidation/biogenesis and cellular energy metabolism (Sparks et al., 2005). In addition it is noteworthy that in skeletal muscle of obese subjects the activity of CPT-1 and citrate sythase, two key mitochondrial enzymes, is reduced (Kim et al., 2000). The impaired mitochondrial oxidative capacity can then lead to accumulation of toxic lipid derivatives, such as DAG and ceramide, which, as mentioned earlier, impair insulin signalling (Bruce et al., 2009). There is evidence that ceramide itself contributes to mitochondrial stress. Indeed, ceramide has been found to alter membrane permeability, inhibit electron transport chain intermediates and induce oxidative stress (Bikman and Summers, 2011). The finding that inhibition of *de novo* ceramide synthesis in high-fat diet fed mice enhances citrate synthase activity and oxygen consumption is consistent with this latter view (Ussher et al., 2010). SFA-induced inflammation can also impair mitochondrial function. Indeed, cells exposed to cytokines such as TNF-α, IL-1β and IL-6 show decreased mitochondrial membrane potential, decreased cellular ATP production and increased intracellular ROS levels (Ji et al., 2011; Yasuhara et al., 2005).

Fatty acid-induced insulin resistance is also associated with production of ROS and oxidative stress. ROS are produced mainly at complex I and III in mitochondria and under normal circumstances can regulate several biological processes including, for example, immune responses, cell adhesion and growth factor/hormone action (Martins et al., 2012). However, high levels of ROS are negatively associated with atherosclerosis, neurodegenerative diseases,

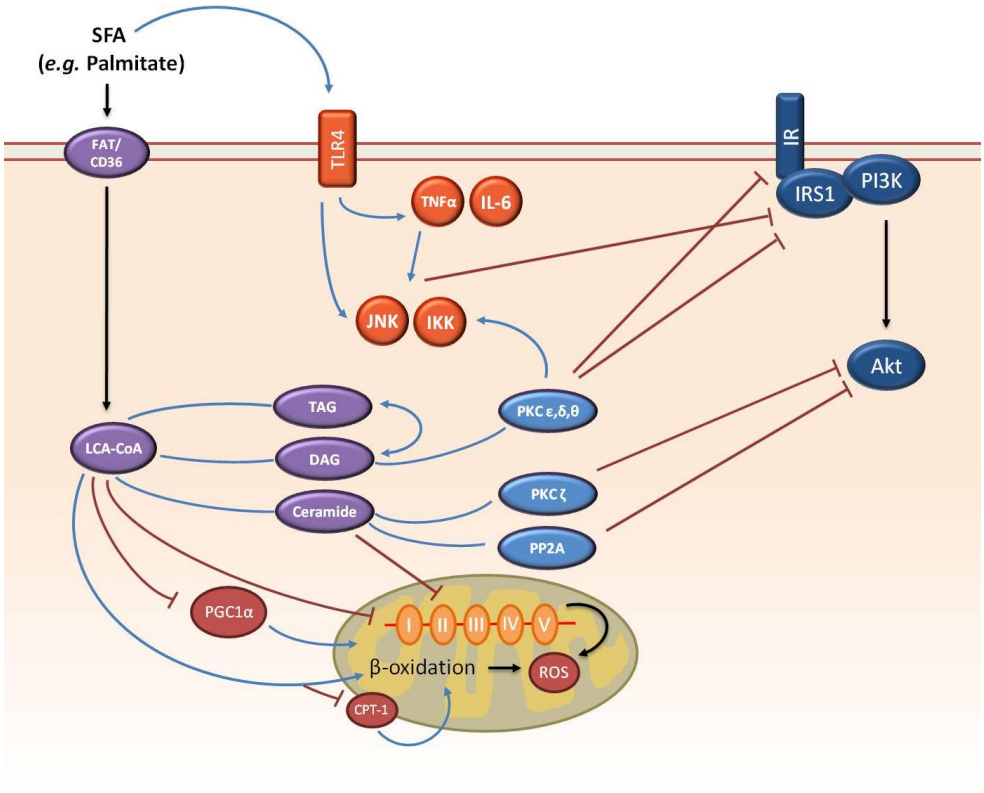


Figure 1.9 Mechanisms contributing to SFA-induced insulin resistance.

Increased levels of saturated fatty acids result partially in raised triacylglycerol storage but mainly in increased concentration of lipid derivatives, such as DAG and ceramide. DAG impairs insulin signalling by activation of novel PKCs, which inhibit IR and IRS1 directly by phosphorylation of serine residues and indirectly by activation of JNK and IKK. These proinflammatory kinases are activated as a consequence of SFA-induced TLR4 activation, which induces secretion of proinflammatory cytokines, such as TNFα and IL-6, promoting a low-grade inflammatory state. Ceramide promotes the activation of PKCζ and PP2A, both of which inhibit Akt through different mechanisms. Ceramide also contributes to mitochondrial stress and dysfunction by altering membrane permeability and inhibiting the electron transport chain. Mitochondrial stress is also promoted by impaired fatty acid oxidation that leads to increased production of ROS. SFAs have also been shown to decrease the expression of PGC1α, mitochondrial complexes I, II, III and IV and to impair the activity of CPT-1.

obesity-induced insulin resistance and type 2 diabetes (Bonnard et al., 2008; Martins et al., 2012). Studies in diet-induced obese mice demonstrate enhanced expression of inducible nitric oxide synthase (iNOS) and production of reactive nitrogen species (RNS) in skeletal muscle (Pilon et al., 2010). The notion that ROS and RNS are associated with decreased insulin sensitivity is supported by the finding that mice lacking iNOS are protected by diet/obesity-induced insulin resistance in skeletal muscle (Dallaire et al., 2008; Perreault and Marette, 2001). Indeed, it has been shown that treating L6 myotubes with H_2O_2 lowers insulin-stimulated glucose uptake (Maddux et al., 2001), while exposure of rat soleus muscle to nitric oxide donors reduces insulin-mediated activation of IR, IRS and Akt (Carvalho-Filho et al., 2005). In skeletal muscle ROS are mainly produced in mitochondria, consequently it follows that mitochondria DNA, protein and lipids can be affected by irreversible modification induced by these metabolites, thus resulting in impaired mitochondrial function (Indo et al., 2007).

1.7 Unsaturated fatty acids (UFAs) and their effect on insulin signalling

In addition to the dietary fat intake numerous studies have shown that the quality of fat is also a crucial factor in the development of insulin resistance and metabolic syndrome (Coll et al., 2008; Dimopoulos et al., 2006; Yuzefovych et al., 2010). Kinsell *et al.* were probably the first to suggest that SFAs and UFAs could exert different effects on insulin activity in diabetic patients (Kinsell et al., 1959). Indeed, as already discussed above, high levels of SFAs are implicated strongly in the development of insulin resistance, whereas monounsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acids do not display overt pathogenic effect and, if anything, confer protection against the several detrimental effect of SFAs (Coll et al., 2008; Fedor and Kelley, 2009;

Kalupahana et al., 2011; Kwon et al., 2014; Salvado et al., 2013; Yuzefovych et al., 2010).

1.7.1 Monounsaturated fatty acids (MUFAs)

The important role of MUFAs in preventing insulin resistance has been determined by the KANWU study. In this study, 162 healthy men and women were followed for three months on a controlled isoenergetic diet either rich in SFAs or MUFAs. Insulin sensitivity was reduced by 10% on the high-SFA diet while it was enhanced on the MUFA diet, with no effect on insulin secretion (Vessby et al., 2001). However, the benificial effect of MUFAs was not seen in individuals with a high fat intake (>37% of energy), suggesting that both amount and quality of the fat were important in the modulation of insulin action (Vessby et al., 2001). There are several mechanisms that explain the beneficial effect of MUFAs. First, MUFAs preferentially accumulate as TAG due to affinity of DGAT for UFAs, while SFAs are mainly incorporated into DAG and ceramide, which induce insulin resistance (Bergouignan et al., 2009). In addition, co-provision of oleic acid, the most common and studied MUFA, to palmitate-treated human skeletal muscle cells has been shown to promote TAG accumulation, thus preventing DAG production and associated PKC activation (Pickersgill et al., 2007). Moreover, MUFAs are more rapidly oxidised than long-chain SFAs (DeLany et al., 2000) and can prevent insulin resistance by channeling palmitate towards mitochondrial β-oxidation thereby reducing accumulation of lipotoxic lipid intermediates (Coll et al., 2008). The ability to stimulate β-oxidation arises, in part, because MUFAs act as ligands and activators of PPARα, a transcription factor responsible for expression of genes encoding proteins involved in fatty acid oxidation, such as PGC1α and CPT-1 (Kliewer et

al., 1997). Whilst palmitate suppresses expression of both PGC1α and CPT-1 in muscle cells, oleic acid antagonises the effect of palmitate on both genes. As a consequence mitochondrial respiration is mantained and this helps counter insulin resistance (Coll et al., 2008; Yuzefovych et al., 2010). It is noteworthy that oleic acid supplementation also has the ability to protect skeletal muscle cells against palmitate-induced oxidative stress, apoptosis, JNK-mediated pro-inflammatory signalling and impaired insulin sensitivity (Yuzefovych et al., 2010). In addition to oleic acid, palmitoleate also enhances insulin sensitivity by promoting basal glucose uptake in muscle cells *via* increased translocation of GLUT1 and GLUT4 (Dimopoulos et al., 2006) and prevents palmitate-induced pro-inflammatory signalling and mitochondrial dysfunction (Macrae et al., 2013).

1.7.2 Polyunsaturated fatty acids (PUFAs)

As well as MUFAs, PUFAs have been linked to modulation of insulin sensitivity, although there are differences in term of benefits between n-3 and n-6 PUFAs. Linoleic acid represents the most abundant n-6 PUFA and a diet enriched in this and other n-6 PUFAs has been shown to improve insulin sensitivity after only 5 weeks (Summers et al., 2002). Moreover, rats fed a diet enriched with n-6 PUFAs show improved glucose tolerance and enhanced partitioning into TAG compared to DAG in skeletal muscle (Lee et al., 2006). Similarly, *in vitro* studies indicate that incubation of palmitate-treated L6 myotubes with linoleic acid alleviates insulin resistance by greater partitioning of palmitate into TAG (Lee et al., 2006).

Of the n-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are found mainly in fish, have a primary role in the modulation of insulin sensitivity (Fedor and Kelley, 2009; Kalupahana et al., 2011; Li et al.,

2008). Replacement of only 6% of n-6 PUFAs and SFAs in a high-fat diet (60%) with fish oil rich in n-3 PUFAs effectively prevented insulin resistance in skeletal muscle and liver of rat (Storlien et al., 1987). Furthermore, substitution of fish oil with SFAs over a three week period in healthy young subjects induced (i) a 40% decrease in insulinaemic response with no change in the glycaemic response and (ii) a 35% increase in fat oxidation after oral glucose load; responses that are indicative of improved insulin sensitivity (Delarue et al., 1996).

The beneficial effect of both n-3 and n-6 PUFAs is mainly due to their modulation of lipid metabolism. By direct interaction with PPARs, PUFAs can enhance the expression of genes encoding proteins involved in fatty acid transport and oxidation (Clarke, 2000), and mitochondrial biogenesis (Clarke, 2000), such as fatty acid binding proteins (Kletzien et al., 1992), fatty acid transporter (Martin et al., 1997), CPT-1 and PGC1α (Feillet-Coudray et al., 2013; Flachs et al., 2005). On the other hand, PUFAs suppress hepatic lipogenesis by decreasing the expression of ACC, with consequent reduction of malonyl-CoA levels and fatty acid synthase *via* a reduction in the liver expression of sterol regulatory element binding protein-1 (SREBP-1); a key transcription factor that regulates lipid homeostasis (Toussant et al., 1981; Wilson et al., 1990; Xu et al., 1999). In addition, studies performed in both cultured human skeletal muscle cells and obese mice have reported the ability of PUFAs to increase GLUT4 expression thus enhancing uptake and metabolism of glucose with associated benefits upon insulin sensitivity (Mikami et al., 2012; Vaughan et al., 2012).

Recent work has started to dissect how the ratio of n-3 and n-6 provided in a diet influences health and metabolic processes. Indeed, it is believed that

humans have evolved on a diet with a ratio of n-6 to n-3 of ~1, but that the present day Western diet provides a ratio ~15:1 and that this is associated with cardiovascular, inflammatory and autoimmune diseases (Simopoulos, 2002). Despite the beneficial insulin sensitising effects mentioned above, many n-6 PUFAs, especially arachidonic acid, are substrates for cyclooxygenase and lipooxygenase that catalyse the synthesis of eicosanoids, which propagate inflammatory signalling. This high levels of n-6 PUFAs or a high-n-6/n-3 ratio in the diet contributes to the development of inflammatory and immune diseases, whereas increased intake of n-3 PUFAs confers protective effects (Sijben and Calder, 2007).

1.8 PP2A

PP2A is a ubiquitously expressed serine/threonine phosphatase, which represents 0.3-1% of the total cellular protein in mammalian cells (Ruediger et al., 1991). PP2A is a multi functional phosphatase with more than thirty substrates, of which Akt, PKC, p70S6K, cAMP dependent kinases, CaM-kinases and ERK/MAP kinases are notable targets (Millward et al., 1999). It follows that it is involved in the regulation of several cellular processes, such as cell metabolism, cell cycle, DNA replication, cell proliferation, transcription, translation, signal transduction, cell mobility, apoptosis and cancer (Alberts et al., 1993; Glenn and Eckhart, 1993; Schonthal, 2001; Tung et al., 1985).

1.8.1 PP2A structure

Different holoenzyme complexes of PP2A have been identified and characterised in a variety of tissues. The PP2A complex is present *in vivo* as a dimeric ($PP2A_D$) or trimeric ($PP2A_T$) form. $PP2A_D$ consists of a catalytic subunit

(PP2Ac) of ~36kDa bound to a scaffolding subunit (PR65/A) of ~65 kDa. This dimeric complex can then interact with a regulatory B subunit, which stabilises the holoenzyme. Four different families of regulatory subunits have been identified so far and each of them seems to influence the substrate specificity and the cellular localization of PP2A (Table 1.2) (Mayer-Jaekel and Hemmings, 1994; Zolnierowicz, 2000). The catalytic subunit is present in mammalian cells in two isoforms, α and β, which are ubiquitously expressed and share 97% identity (Khew-Goodall et al., 1991). However, mice lacking the PP2Acα die during the embryonic state demonstrating that despite the high similarity, the two isoforms do not have redundant functions (Gotz et al., 1998). The second constituent of $PP2A_D$ is the PR65/A subunit, which, like the catalytic subunit, exists as two different isoforms (α and β) in mammalian cells. These isoforms share 86% identity and are ubiquitously expressed (Hemmings et al., 1990). The structure of PR65/A is characterised by 15 tandem leucine rich repeats of 39-amino-acid sequence, known as HEAT (huntingtin/elongation/A subunit/TOR) motifs. HEAT- motifs are stacked parallel to each other, creating a surface for protein-protein interaction between PR65/A and the catalytic and regulatory subunits (Groves et al., 1999; Hemmings et al., 1990).

1.8.2 PP2A regulation

Considering the role that PP2A plays within reciprocal control of cell signalling events regulated by phosphorylation, it is not surprising that it is itself subject to a tight regulation. PP2A can regulate its own abundance *via* an auto-inhibition mechanism during translation. PP2A can dephosphorylate specific proteins that form part of the translational apparatus, resulting in the inhibition of its mRNA

B subunit family	**Isoforms**	**Tissue expression**	**Subcellular localization**
B/PR55	α	Widely distributed	Cytosolic fraction
	β	Brain	Cytosolic fraction
	γ	Brain	Cytoskeletal fraction
	δ	Widely distributed	Cytoplasm
B'/PR61	α	Heart and skeletal muscle	
	β	Brain	Cytoplasm
	γ	Heart and skeletal muscle	Nucleus
	δ	Brain	Nucleus and cytoplasm
	ε		Cytoplasm
B''/ PR72	PR72	Heart and skeletal muscle	
	PR130	Widely-distributed	
	PR59	Widely-distributed but absent in skeletal muscle	
	PR48		Nucleus
B'''/PR93/PR110	PR93		Nucleus
	PR110		Postsynaptic densities of neuronal dendrites

Table 1.2. Tissue distribution and subcellular localisation of PP2A regulatory subunits.

translation thereby maintaining control of cellular PP2A abundance (Baharians and Schonthal, 1998).

Another level of control of PP2A activity involves post-translational modification of the catalytic subunit on the highly conserved C-terminal domain (304TPDYFL309) (Fig 1.10). PP2Ac can be reversibly phosphorylated on a tyrosine residue (Y307) leading to inactivation of the phosphatase. *In vitro,* the transient phosphorylation of Y307 is mediated by both receptor tyrosine kinases, such as epidermal growth factor and insulin receptors, or non receptor tyrosine kinases such as $pp60^{v\text{-}srk}$, $pp56^{lck}$, Lyn, Fyn and Src (Chen et al., 1992; Chen et al., 1994). The PP2A inhibitor okadaic acid can enhance phosphorylation of PP2Ac suggesting that PP2A can also act as a phosphotyrosine phosphatase and mediate autodephosphorylation as a means of self reactivation (Chen et al., 1992). *In vivo* transient phosphorylation of Y307 is promoted in response to IL-1 and TNFα (Guy et al., 1995), epidermal growth factor, serum (Chen et al., 1994) and insulin (Begum and Ragolia, 1996; Srinivasan and Begum, 1994). It is possible that such reversible inhibition of PP2A works as an accelerating factor during the transmission of signals initiated from receptors to their downstream cellular effectors. PP2Ac can also be phosphorylated *in vitro* on an unidentified threonine residue by an autophosphorylation-activated protein kinase, resulting in the inactivation of both phosphoserine/threonine and phosphotyrosine phosphatase activity (Damuni et al., 1994; Guo and Damuni, 1993).

In addition to phosphorylation, the C-terminal end of PP2Ac can also be methylated. Reversible methylation occurs on the carboxyl group of L309 (Lee et al., 1996; Xie and Clarke, 1994). Two enzymes are involved in the regulation

of this process: the leucine carboxyl methyl transferase-I (LCMT-I) (De Baere et al., 1999) and the PP2A methylesterase-1 (PME-1) (Ogris et al., 1999). L309 methylation can affect PP2A holoenzyme assembly. This notion is supported by the finding that inactivation of LCMT-1 inhibits the formation of $PP2A_T$ (Wu et al., 2000) and that methylated L309 is required for the association of the B/PR55α subunit to the catalytic subunit (Bryant et al., 1999). Okadaic acid can inhibit PP2A not only by increasing its phosphorylation but also by reducing its methylation by binding to the catalytic subunit and therefore preventing the interaction of LCMT-1 to the C-terminal part of PP2Ac (Li and Damuni, 1994).

1.8.3 PP2A role in cellular signalling

PP2A plays a major role in the regulation of several cellular processes including cell cycle, apoptosis and mRNA translation (Zolnierowicz, 2000). The phosphatase can regulate the activities of numerous kinases, such as Akt. Such regulation is significant as aberrant modulation of Akt-directed insulin signalling by PP2A could promote insulin resistance as seen in response to sustained increases in SFA availability. Palmitate, a SFA, can drive synthesis of ceramide, which is a potent activator of PP2A and, as such, contributes to enhanced dephosphorylation/inactivation of Akt and thus insulin signalling (Galbo et al., 2011). In addition to Akt, PP2A can also modulate the ERK/MAPK kinase pathway. Both ERK1/2 and MEK1 have been identified as PP2A substrates (Gomez and Cohen, 1991) and treatment with either okadaic acid or SV40 small-t antigen, which inhibit the phosphatase, results in the activation of these kinases (Gause et al., 1993; Sonoda et al., 1997; Sontag et al., 1993). PP2A is also involved in the modulation of mRNA translation. P70S6K, which plays a key role in control of translation, has been found to form a stable complex with

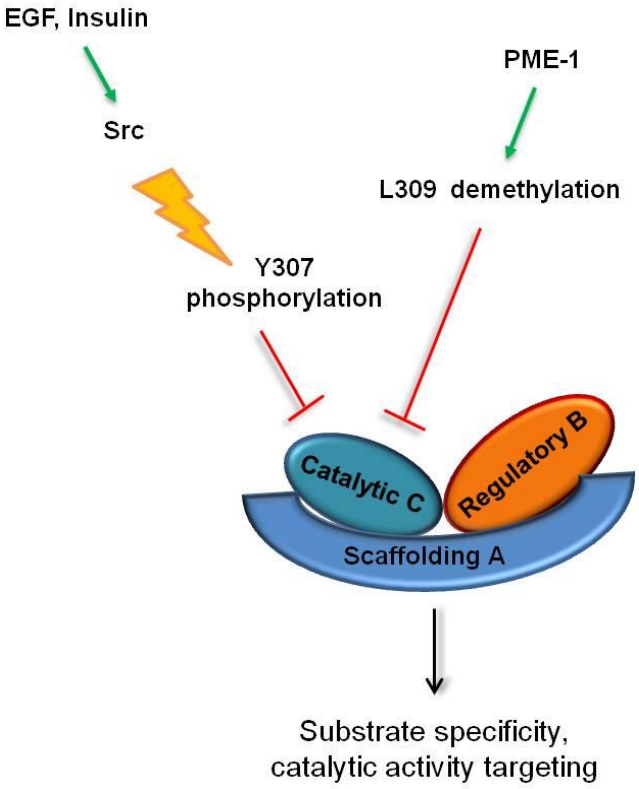


Figure 1.10 Structure and regulation of PP2A.

PP2A complex consists of a catalytic subunit (C), a scaffolding subunit (A) and a regulatory subunit (B). The activity of PP2A depends on post translational modification of its catalytic subunit. Demethylation of the L309 residue by PP2A methylesterase-1 (PME-1) and phosphorylation of Y307 residue by insulin/grow factor induced-Src result in the inactivation of the phosphatase.

PP2A *in vivo* (Westphal et al., 1999) and evidence exists showing that P70S6K can be dephosphorylated by the purified phosphatase *in vitro* (Ballou et al., 1988). The protein Tap42/α4 (type 2A-associated protein of 42 kDa) can directly interact with and inhibit the catalytic subunit of PP2A. This interaction is disrupted by rapamycin, an inhibitor of mTOR, suggesting that PP2A may participate in the rapamycin-sensitive pathway that links extracellular stimuli with mRNA translation (Murata et al., 1997). PP2A is also involved in the termination of translation since it can interact with the eukaryotic release factor 1 (eRF1), which may help bring the phosphatase in proximity of putative ribosomal targets belonging to the translation apparatus (Andjelkovic et al., 1996b; Lechward et al., 1999). There is also evidence that this phosphatase is connected to apoptosis, since the scaffolding subunit A/PR65 has been identified as a substrate for caspase 3. Caspase 3 is responsible for the cleavage of key enzymes involved in DNA repair (Cryns et al., 1996). By cleaving the A/PR65 subunit, caspase 3 enhances the phosphatase activity of the free catalytic subunit, which can then act on its substrates like MAPK (Santoro et al., 1998). In addition, PP2A can dephosphorylate Bcl-2 on its S70 residue. Bcl-2 requires this phosphorylation in order to exert its anti-apoptotic effect (Deng et al., 1998). Several studies have implicated PP2A in the regulation of the cell cycle, especially in the G2/M transition. This transition is mediated by cyclin dependent kinase 1 (Cdk1/Cdc2), which forms a complex with cyclin B. Cdc2 activity requires it to be phosphorylated on T161 and PP2A has been identified *in vitro* as the phosphatase responsible for dephosphorylation of this residue. T14 and 15 are two inhibitory residues of Cdc2 and their phosphorylation is regulated by the kinase WEE1 and the dual-

specificity phosphatase cell division cycle 25 (CDC25). PP2A can indirectly inhibit Cdc2 by activating WEE1 kinases and inactivating the CDC25 phosphatase (Clarke et al., 1993; Mueller et al., 1995; Yamashita et al., 1990). There is also evidence of PP2A involvement in inflammation (DiDonato et al., 1997; Sun et al., 1995) and carcinogenesis (MacKintosh and MacKintosh, 1994; Wang et al., 1998).

1.9 An alternative source of energy: amino acids

As mentioned earlier, cells can handle different types of fuel depending on nutrient availability and tissue need. Whilst glucose and fatty acids represent the primary fuel sources, amino acids also represent a potential energy source and their pool size requires to be strictly regulated by cells *via* modulation of their transport and metabolism.

1.10 Amino acids classification

Amino acids represent the building blocks of proteins. However, of the three hundred amino acids present in nature, only twenty actually constitute components of proteins. Most microoorganisms can synthesise all twenty amino acids, but mammals cannot synthesise nine of them. These are known as essential amino acids and have to be consumed in the diet, whereas the remaining amino acids are designated as non essential and can be synthesised in a small number of steps (Berg, 2002). Due to variations in the side chain (R-groups) each amino acid has different functions and chemical properties (Berg, 2002). Table 1.3 shows a classification based on polarity and charge of the R-group.

Amino acid	**Abbreviation**	**Single letter code**	**Side-Group polarity**	**Side-group charge**
Arginine	Arg	R	polar	positive
Asparagine	Asn	N	polar	positive
Aspartate	Asp	D	polar	negative
Cysteine	Cys	C	polar	neutral
Glutamate	Glu	E	polar	negative
Glutamine	Gln	Q	polar	neutral
Histidine	His	H	polar	positive
Lysine	Lys	K	polar	positive
Serine	Ser	S	polar	neutral
Threonine	Thr	T	polar	neutral
Tyrosine	Tyr	Y	polar	neutral
Alanine	Ala	A	non-polar	neutral
Glycine	Gly	G	non-polar	neutral
Isoleucine	Ile	I	non-polar	neutral
Leucine	Leu	L	non-polar	neutral
Methionine	Met	M	non-polar	neutral
Phenylalanine	Phe	F	non-polar	neutral
Proline	Pro	P	non-polar	neutral
Tryptophan	Trp	W	non-polar	neutral
Valine	Val	V	non-polar	neutral

Table 1.3 Nomenclature and chemical features of the 20 major Amino Acids.

1.11 Amino acid sensing

Cells are able to ‘sense’ and respond to changes in extracellular nutrients by functional adaptations, such as up or down regulation of genes involved in metabolism or nutrient transport in order to maintain normal cellular homeostasis. Such adaptation involves nutrient sensors such as receptors, transporters and enzymes, which initiate or affect signalling cascades responsive to changes in the nutrient environment (Taylor, 2014). Two main intracellular amino acid sensing-signalling pathways have been identified in mammalian cells: the mTORC1 and the general control non-derepressible 2 (GCN2) pathways. While mTORC1 pathway is activated in response to amino acids sufficiency to regulate protein synthesis and cell proliferation, GCN2 is stimulated by amino acid insufficiency to induce expression of genes that allow cells to adapt to amino acid deprivation (Fig 1.11) (Taylor, 2014).

1.11.1 mTOR

TOR (target of rapamycin, mTOR in mammals) is a conserved atypical serine/threonine kinase of ~290 kDa which plays a crucial role in the regulation of cell growth and proliferation. As suggested by the name, TOR is sensitive to rapamycin, a *Streptomyces hygroscopicus* derived fungicide which has been discovered to have anti-proliferative properties (Heitman et al., 1991; Tsang et al., 2007; Vezina et al., 1975). mTOR is present as two functionally distinct complexes: mTORC1 and mTORC2. mTORC1 is rapamycin sensitive whereas mTORC2 is insensitive to the drug and can be activated by growth factors leading to phosphorylation of Akt on S473 and regulation of cytoskeletal organisation (Jacinto et al., 2004). mTORC1 can be activated by both growth

factors and amino acids and plays a major role in the regulation of protein synthesis and cell growth. mTOR is the catalytic subunit of the mTORC1 complex, which also consists of a scaffolding protein Raptor (regulatory-associated protein of mTOR) that is responsible for substrate recognition; the positive regulator mLST8 (mammalian lethal with sec 13 protein 8), and the inhibitory modulators PRAS40 (proline-rich Akt substrate 40 kDa) and Deptor (Dep-domain mTOR interacting protein) (Kim et al., 2013). The two best-characterised substrates of mTORC1 are P70S6K and eIF4E-binding protein 1 (4EBP1), which are involved in the regulation of protein synthesis (Kim et al., 2002). Under nutrient-rich conditions, amino acids induce a conformational change in the mTORC1 complex promoting mTOR interaction with P70S6K and 4EBP1 *via* Raptor and their phosphorylation (Kim et al., 2002). Phosphorylation of 4EBP1 results in its inhibition and release of the eukaryotic translation initiation factor, eIF4E, which is involved in directing ribosomes to the cap structure of mRNA to promote cap-dependent translation (Proud, 2002). Raptor recognises P70S6K *via* its TOS (TOR-signalling) motif and allows its interaction with mTOR (Schalm et al., 2005). P70S6K regulates translation through its downstream targets ribosomal protein S6 and eukaryotic translation initiation factor (eIF4A). Ribosomal protein S6 is a component of the 40S ribosome subunit involved in translation initiation at the 5’ 7-methylguanylate cap of mRNA. Phosphorylation by P70S6K enhances S6 cap-binding activity improving protein synthesis (Ruvinsky and Meyuhas, 2006). As indicated earlier, amino acids can stimulate mTOR activation, an event potentially involving a number of different mechanisms. First, amino acids have been shown to enhance the activity of the vacuolar protein sorting 34 (Vps34), a

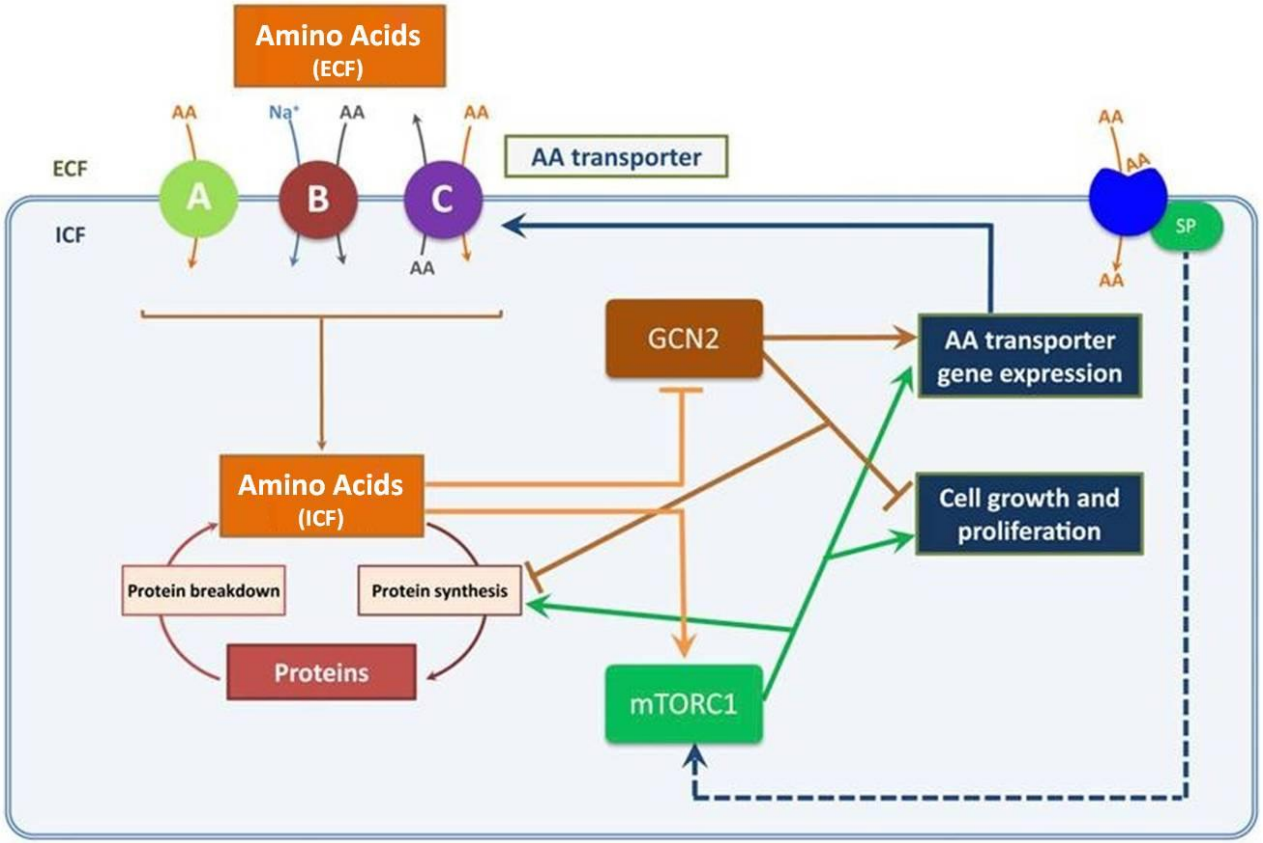


Figure 1.11 Amino acids sensing signalling pathways.

The 2 major amino acids (AA)-sensitive signaling pathways in mammalian cells are GCN2 and mTORC1**,** which respond to changes in AA availability and regulate protein turnover. Modified from (Taylor, 2014). Intracellular fluid, ICF; Extracellular fluid, ECF; signaling peptide , SP.

phosphatidylinositol 3-kinase that appears to participate in nutrient-induced mTOR activation (Gulati et al., 2008). Indeed, silencing of human Vps34 has been shown to inhibit amino acid-induced activation of P70S6K, whilst its overexpression leads to activation of its mTOR downstream target (Nobukuni et al., 2005). mTORC1 activation also involves small GTPases. The Ras-related GTPase (Rag) family consists of four members, A, B, C and D, which exist as heterodimers of RagA/B and RagC/D (Shaw, 2008). The combination of RagA/B-GTP and RagC/D-GDP represents the active form while the inverse nucleotide association represents the inactive one (Duran and Hall, 2012). Rag GTPase heterodimers interact with a protein termed the Ragulator that is localised on the lysosome membrane (Bar-Peled et al., 2012). Amino acids stimulate a direct interaction between the active form of the Rag GTPases and Raptor, leading to localisation of the mTORC1 complex to the lysosome surface. This event allows mTORC1 to interact with Ras homologue enriched in brain (Rheb), a small GTPase that binds to the N-terminus of the mTOR catalytic domain resulting in mTORC1 activation (Long et al., 2005). Rheb-GTP loading is itself regulated by the TSC1/2, a Rheb GAP that induces the conversion of Rheb-GTP to Rheb-GDP leading to its inhibition (Inoki et al., 2003).

1.11.2 GCN2

While amino acid provision activates the mTORC1 pathway, amino acid deprivation induces an amino acid response (AAR) signal transduction pathway (Kilberg et al., 2009). This pathway is mediated by GCN2 that acts as a nutrient sensor by binding to uncharged tRNAS, whose levels are increased by amino acid limitation (Dong et al., 2000). Once activated by this interaction, GCN2

phosphorylates the translation initiator factor eIF2α on S51, leading to its inhibition and consequent reduction of protein synthesis (Dong et al., 2000). Paradoxically, eIF2α phosphorylation results in increased translation of a group of stress-responsive mRNAs, such as activating transcription factors (ATF) 4 and 5 (Vattem and Wek, 2004; Zhou et al., 2008) and growth arrest and damage-inducible 34 (GADD34) (Lee et al., 2009). ATF4 is a transcription factor with ubiquitous distribution but very low expression. This is due to two upstream open reading frames present in the 5'UTR of ATF4 mRNA, which inhibits its translation in normal conditions but induces it under stress conditions, such as amino acid deprivation (Harding et al., 2000). ATF4 promotes the transcription of genes encoding for proteins involved in the regulation of stress response, such as the amino acid transporters CAT-1 (Cationic amino acid transporter 1) and SNAT2 (System A/sodium-coupled neutral amino acid transporter), the regulator of cell growth TRB3 (Tribbles homolog 3) and the transcription factor CHOP (C/EBP homology protein) (Kilberg et al., 2009). ATF4 induces the transcription of these genes by binding to CCAAT-enhancer binding protein-activating transcription factor (C/EBP-ATF) response elements (CARE), which can act as amino acid response elements (AARE) but also mediate the transcription of these genes under ER stress (Barbosa-Tessmann et al., 2000).

1.12 Amino acid transport

Cellular supply of amino acids to meet the demands for protein synthesis can be met both by biosynthesis and transport of amino acids into the cells. The transport of amino acids across the membrane is a tightly regulated process, which responds to several stimuli, such as substrate availability, stress and hormones and is mainly catalysed by transporters characterised as being,

primary, secondary or tertiary active transporters (Fig 1.12) (Hundal and Taylor, 2009). Primary active transporters utilise ATP hydrolysis to help mediate the transport of solutes against their electrochemical gradient. The Na^+/K^+ pump belongs to this category and is capable of ATP hydrolysis to promote the efflux of $3Na^+$ ions in exchange for $2K^+$ ions. This process contributes to maintenance of a membrane potential and a transmembrane ion gradient that can be utilised to drive secondary active transporters (*e.g.* SNAT2). Most of these transporters bind amino acid substrates and Na^+, and use the electrochemical gradient of the latter to translocate both substrates across the membrane. The concentration gradient produced by secondary active transport can then be utilised by tertiary active transporters (*e.g.* System L/L-type amino acid transporter 1 (LAT1)) as a driving force for counter-exchange of extracellular solutes for those resident in the intracellular compartment (Hundal and Taylor, 2009). Christensen *et al.* first classified amino acid transporters as “systems” by identifying those systems that accepted structurally related amino acids (Christensen, 1984). By this process, different systems have been identified for the transport of neutral, cationic and anionic amino acids (Table 1.4).

1.13 Role of amino acid transporters in amino acid sensing: the transceptor concept

It is widely known that amino acids influence several cell functions including, for example, regulation of cell signalling gene expression and metabolism (Hyde et al., 2003). As mentioned earlier, changes in amino acid availability can lead to activation of the GCN2/ATF4 pathway, resulting in expression of genes encoding amino acid transporters and amino acid biosynthetic enzymes and stimulation of the mTORC1/P70S6K axis, which enhances cell growth and

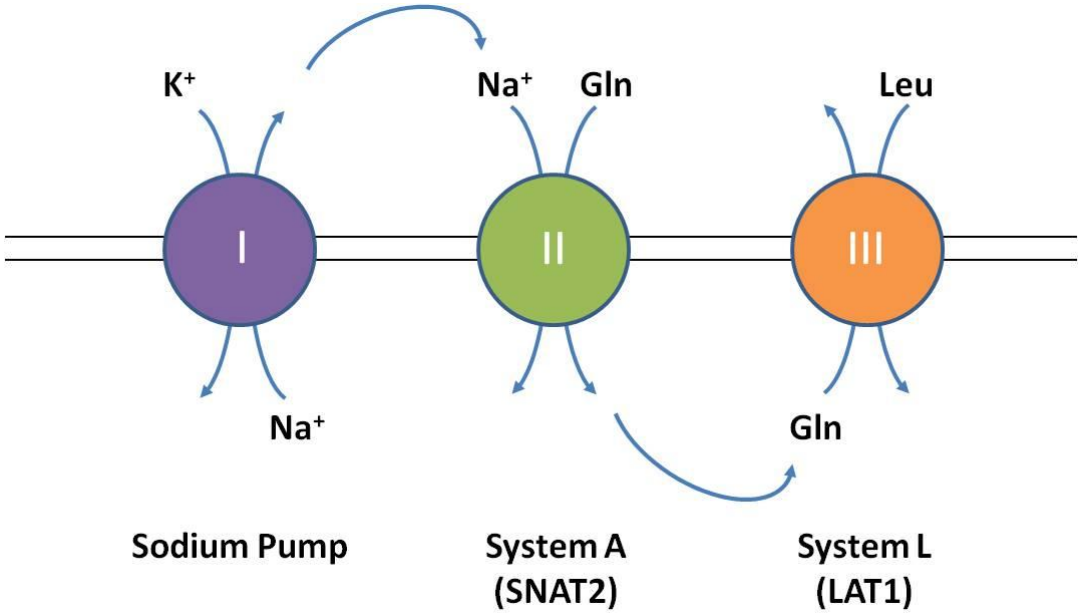


Figure 1.12 Integration of primary (I), secondary (II) and tertiary (III) active transport systems.

Secondary active amino acid transport is driven by the Na^+/K^+ pump, the primary active transporters. Secondary active transporters, such as System A, generate symport of small neutral amino acids along with movement of sodium down its electrochemical gradient into cells; these amino acids can then be used as substrates for tertiary active transporters, such as System L, which promote amino acid exchange.

(ai) Neutral-amino-acid transporters: sodium-dependent

System	Protein	Gene	Amino acid substrates (one-letter code)	Notes
A	SAT1	SLC38A1	G, A, S, C, Q, N, H, M, T, Me-AIB, P, Y, V	Short-chained-neutral-amino-acid transport.
	SAT2	SLC38A2	G, P, A, S, C, Q, N, H, M, Me-AIB	Sensitive to low pH. Ubiquitous expression.
	SAT3	SLC38A4	G, P, A, S, C, N, M, H, K, R	SAT3 may also function as a Na^+-independent cationic amino acid transporter.
ASC	ASCT1	SLC1A4	A, S, C	High-affinity short-chain-amino-acid exchanger.
	ASCT2	SLC1A5	A, S, C, T, Q	Ubiquitous expression.
B^o	ASCT2	SLC1A5	A, S, C, T, Q, F, W, Y	Broad substrate specificity. Expressed on apical surface of many epithelia. May include novel isoforms of ASCT2 (e.g. a novel gene recently characterized by Pollard et al. [216]).
BETA	GAT1	SLC6A1	GABA	Widely expressed Cl^--dependent GABA, betaine and taurine transporters.
	GAT2	SLC6A13	GABA, betaine, P, β-A	
	GAT3	SLC6A11	GABA, betaine, taurine	
	BGT1	SLC6A12	GABA, betaine	
	TAUT	SLC6A6	Taurine	
Gly	GLYT1	SLC6A9	G, sarcosine	Na^+- and Cl^--dependent high-affinity glycine transport.
	GLYT2	SLC6A5	G, sarcosine	Expressed in brain and some non-neural tissues.
IMINO	–	–	P	Na^+-dependent eptihelial proline transporter, inhibited by Me-AIB.
N	SN1	SLC38A3	Q, N, H	Li^+-tolerant transport of Gln, Asn and His. H^+ antiport.
	SN2	SLC38A5	Q, N, H, S, G	Li^+-intolerant variants described
N^m	–	–	Q, N, H	
N^b	–	–	Q, N, H	
PHE	–	–	F, M	Brush-border transporter for Phe and Met
PROT	PROT	SLC6A7	P	Proline transporter in central nervous system.

(aii) Neutral-amino-acid transporters: sodium-independent

System	Protein	Gene	Amino acid substrates	Notes
asc*	Asc1	SLC7A10	G, A, S, C, T	Small neutral AA exchanger.
	Asc2		G, A, S, T	
imino	PAT1/LYAAT1	SLC36A1	P, G, A, β-A, GABA Me-AIB	H^+-coupled transport of small neutral amino acids. Inhibited by Me-AIB.
	PAT2/LYAAT2	SLC36A2	P, G, A, β-A, GABA Me-AIB	
L*	LAT1	SLC7A5	H, M, L, I, V, F, Y, W, Q	Ubiquitously expressed exchanger for large hydrophobic amino acids.
	LAT2	SLC7A8	A, S, C, T, N, Q, H, M, L, I, V, F, Y, W	
T	TAT1	SLC16A10	F, Y, W	Aromatic-amino-acid transporter. H^+/monocarboxylate transporter family – insensitive to pH, however.

(bi) Anionic-amino-acid transporters: sodium-independent

System	Protein	Gene	Amino acid substrates	Notes
X^-_{AG}	EAAT1	SLC1A3	E, D	Widespread Glu and Asp transporter. K^+ antiport.
	EAAT2	SLC1A2	E, D	Substrate-dependent uncoupled anion flux.
	EAAT3	SLC1A1	E, D, C	Lack of stereospecificity toward Asp.
	EAAT4	SLC1A6	E, D	
	EAAT5	SLC1A7	E, D	

(bii) Anionic-amino-acid transporters: sodium-independent

System	Protein	Gene	Amino acid substrates	Notes
x^{-c*}	xCT	SLC7A11	E, Ci	Electroneutral Glu/cystine exchanger.
–	XAT2	–	D, E	Non-functional upon 4F2hc/rbAT heavy-chain co-expression. Predicted to associate with a novel glycoprotein.

(ci) Cationic-amino-acid transporters: sodium-dependent

System	Protein	Gene	Amino acid substrates	Notes
$B^{0,+}$	ATB(o,+)	SLC6A14	K, R, A, S, C, T, N, Q, H, M, I, L, V, F, Y, W	Blastocysts and possibly brush-border membrane. Broad specificity for neutral and cationic amino acids. Accepts BCH.
y^+L^*	y + LAT1 y + LAT2	SLC7A7 SLC7A6	K, R, Q, H, M, L K, R, Q, H, M, L, A, C	Na^+-dependent cationic/neutral-amino-acid exchanger. Electroneutral.

(cii) Cationic-amino-acid transporters: sodium-independent

System	Protein	Gene	Amino acid substrates	Notes
$b^{0,+**}$	b(o,+)AT	SLC7A9	K, R, A, S, C, T, N, Q, H, M, I, L, V, F, Y, W, Ci	Broad-specificity cationic- and neutral-amino-acid exchanger.
y^+	Cat-1 Cat-2 Cat-3 Cat-4	SLC7A1 SLC7A2 SLC7A3 SLC7A4	R, K, H R, K, H R, K Unknown	Cationic-amino-acid (and Na^+-dependent neutral-amino-acid) transport. Variable degree of *trans*-stimulation.

Table 1.4 Mammalian Amino Acid transporters.

(Hyde et al., 2003)

proliferation. Since the activity of amino acid transporters can help modulate the intracellular amino acid pool *via* their gate-keeping functions, they are increasingly thought to play a role also in amino acid sensing and cell signalling (Hundal and Taylor, 2009). It is plausible that transporters could initiate a signal transduction cascade in direct response to changes in substrate loading or by regulating the availability of a specific amino acid, which interacts with an intracellular nutrient sensor(s) and receptor(s) (Hyde et al., 2003). Amino acid transporters that possess both transporter and receptor functions have been termed “transceptors” and have been observed mainly in lower eukaryotes with recent candidates also being identified among the family of mammalian amino acid transporters (*e.g.* SNAT2) (Hundal and Taylor, 2009; Hyde et al., 2007). The first evidence that nutrient transporters can also act as sensors derives

from studies performed on the prokaryotic phosphoenol pyruvate- carbohydrate phosphotrasferase system (PTS) (Postma et al., 1993). Several transceptors have now been identified in eukaryotic systems, all belonging to the amino acid-polyamine-organocation (APC) superfamily of transporters, suggesting that this function has been highly conserved through evolution (Hundal and Taylor, 2009). For example, in *Saccharomyces cerevisiae* the transporter Ssy1p also possesses a sensing function. Binding of the amino acids to Ssyp1 induces a conformational change in the transporter activating a signal transduction cascade that results in the activation of transcription factors that promote expression of amino acid permeases (Hundal and Taylor, 2009). In Drosophila three distinct transporters with relevant effects on cell growth have been identified: Minidiscs (MDN), Slimfast (Slif) and Pathetic (Path). Mutations in MDN result in larvae with growth defects, suggesting the involvement of the transporter in the nutrient/signalling pathway that modulates proliferation (Martin et al., 2000). Mutations in Slif also lead to larvae with growth retardation and morphological problems, mimicking the phenotype obtained by loss of TOR signalling (Zhang et al., 2000). Path over expression induces over growth of the differentiating eye, suggesting its involvement in mediating TOR signalling (Goberdhan et al., 2005). Since *path* has very low transport capacity, it is thought that transporter occupancy provides a stimulus to preserve TOR signalling in response to changes in amino acid availability (Goberdhan et al., 2005). In the mammalian central nervous system the glial glutamate/aspartate transporters GLutamate ASpartate Transporter (GLAST)/ Excitatory Amino Acid Transporter 1 (EAAT1) can sense extracellular glutamate abundance and regulate its clearance. In the presence of high levels of glutamate the binding of

the amino acid to EAAT1 stimulates the recruitment of this transporter to the plasma membrane in astrocyte cultures (Duan et al., 1999). The effects of glutamate upon EAAT1 also influences the surface expression of the ASC-amino acid transporter 2 (ASCT2), a carrier that transports protonated glutamate, suggesting that EAAT1 functions both as a sensor and an effector in the process of clearing excess extracellular glutamate (Gegelashvili et al., 2007). Another mammalian transporter that is thought to act as a transceptor is the Na^+ dependent neutral amino acid transporter SNAT2.

1.14 Na^+ dependent neutral amino acid system: System A/SNAT2

Oxender and Christensen first identified two distinct systems for the transport of neutral amino acids in Ehrlich cells, defined System A and L (Oxender and Christensen, 1963). System A is a secondary active transporter as movement of amino acids is coupled to the downhill movement of Na^+. System A mediates the transport of neutral amino acids containing short, polar or linear side chains but can also promote the uptake of N-methylated substrates, such as 2-methylaminoisobutyric acid (MeAIB) (Christensen et al., 1965). Its activity is reduced at low extracellular pH (Oxender and Christensen, 1963) and by the presence of intracellular substrates, a phenomenon known as trans-inhibition (Christensen, 1975). Moreover, System A can be regulated by hormones such as insulin and by changes in amino acid availability and cell stress (McGivan and Pastor-Anglada, 1994).

1.14.1 SNAT2

Among the subtypes of System A family of carriers, SNAT2 (sodium-coupled neutral amino acid transporter 2), encoded by SLC38A2 gene, is the most

widely expressed (Hatanaka et al., 2000; Sugawara et al., 2000; Yao et al., 2000). SNAT2 is thought to be composed of eleven transmembrane domains with its N-terminus residing in the cytosol and its short C-terminal tail located within the extracellular compartment (Mackenzie and Erickson, 2004). SNAT2 is thought to be glycosylated, since consensus glycosylation sites are present in the predicted extracellular loops of the transporter (Fig 1.13). Work from the Hundal lab, using two antibodies raised against the intracellular N-terminal region and the extracellular loop between transmembrane domains 7 and 8 of SNAT2, revealed that a 60 kDa protein is recognised by both antibodies. Although the predicted molecular size of rat SNAT2 is ~55 kDa, the two glycosylation sites identified on the transporter could alter its mass post-translationally. Interestingly, the antibody against the intracellular N-terminal region detected an additional band of ~40 kDa, which is thought to represent the immature non-glycosylated form of SNAT2 (Hyde et al., 2001). The transporter has also been identified in microsomal and intracellular membranes in rat skeletal muscle cells indicating the presence of an intracellular pool of immature transporters that could be released in response to different stimuli, which are known to enhance System A activity (Hyde et al., 2001). In fact SNAT2 is subject to complex regulatory control mediated by a variety of stimuli including, for example, hyperosmotic shock (Alfieri et al., 2001), amino acid deprivation (Gazzola et al., 2001; Iresjo et al., 2005; Ling et al., 2001; Palii et al., 2004; Yao et al., 2000) and insulin stimulation (Kletzien et al., 1976; McDowell et al., 1998). The effects of such stimuli have been studied in skeletal muscle cells. Kashiwagi *et al*. showed that in L6 rat skeletal muscle cells hyperosmotic shock, induced by adding sucrose to the media for 4 h,

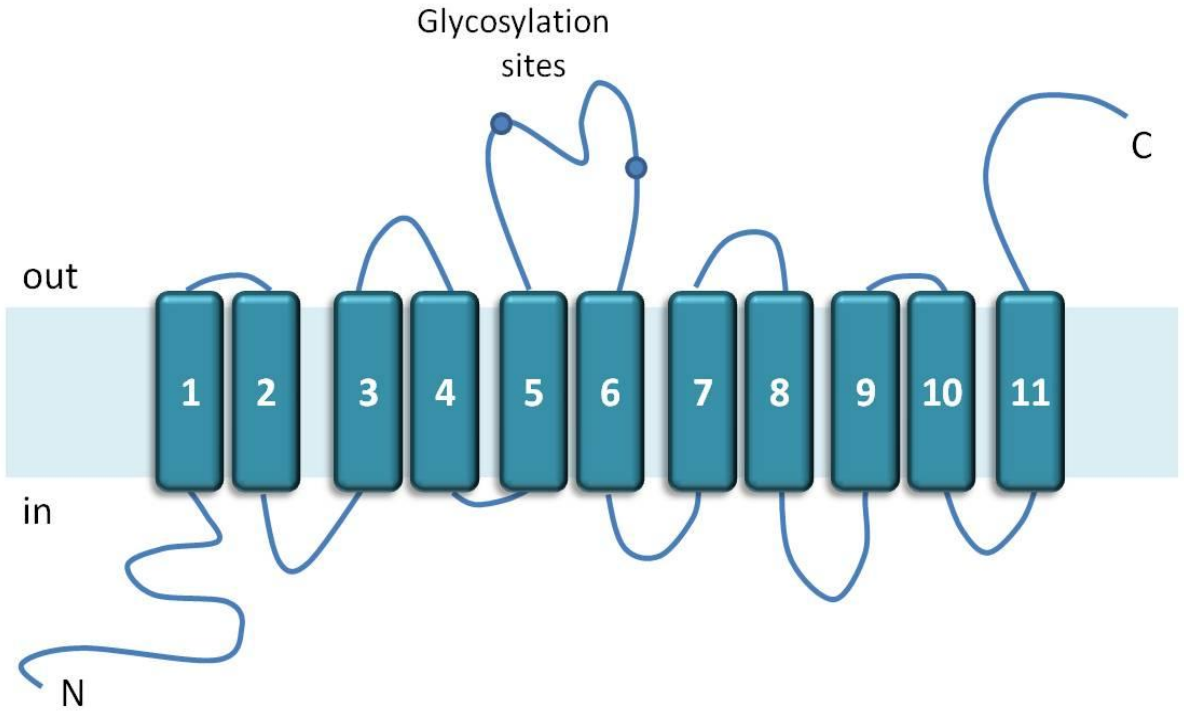


Figure 1.13 Topological structure of SNAT2.

SNAT2 presents a 11 transmembrane domain structure, in which the N-terminus is intracellular and a short C-terminal tail extracellular; consensus glycosylation sites are present in the predicted extracellular loops of the transporter.

upregulated SNAT2 activity by inducing *de novo* synthesis of the transporter (Kashiwagi et al., 2009). This upregulation appears to be mediated by the tonicity enhancer binding protein (TonEBP)/NFAT5 giving that decreased function of this transcription factor resulted in diminished amount of SNAT2 mRNA in transgenic T cells following hypertonic shock (Trama et al., 2002). Insulin stimulation rapidly increases plasma membrane SNAT2 abundance, as it does for the GLUT4 glucose transporter, through its recruitment from an intracellular compartment by a PI3K-dependent mechanism (Hyde et al., 2002; Kashiwagi et al., 2009). Both rapid and long-term upregulation of SNAT2 were observed by amino acid deprivation, a response known as “adaptive regulation”.

1.14.2 SNAT2 adaptive response

The adaptive modulation of SNAT2 is one of the most-studied responses to amino acid withdrawal in mammalian cells. It is a process occurring in two phases: a rapid phase consists of release of the transporter from an intracellular pool and recruitment to the plasma membrane, and a long-term upregulation which involves changes in gene expression *via* activation of the GCN2/ATF4 axis (Kashiwagi et al., 2009; Thiaville et al., 2008). The adaptive response phenomenon was described over 40 years ago by Gazzola *et al.* who showed System A-mediated transport is increased in response to amino acid deprivation in chick embryo heart cells (Gazzola et al., 1972). This increase in transporter activity is prevented by cycloheximide and actinomycin D, which inhibit protein synthesis and gene transcription respectively, suggesting that the adaptive response requires an induction of gene expression. (Gazzola et al., 1972). Subsequently, Palii *et al.* identified an amino acid response element (AARE) in the first intron of the human SNAT2 sequence, which regulates SNAT2 gene

expression in response to amino acid availability (Palii et al., 2004). This AARE is identical to that found in the promoter region of CHOP, a transcription factor that also exhibits adaptive regulation under amino acid withdrawal (Palii et al., 2004). It follows that the increase in SNAT2 expression would partly occur by activation of the GCN2 pathway in response to increased uncharged tRNA levels and consequent transcription of ATF4-dependent AARE containing genes (Thiaville et al., 2008). GCN2 activation also results in the phosphorylation and inhibition of translation initiation factor eIF2α and dephosphorylation of eIF4E, which recognises the 7-methylguanylate cap of mRNA (Wek et al., 2006). Although the translation of the majority of cellular mRNAs is decreased by these events, a pool of mRNAs that encode proteins essential for the cellular stress response are endowed with internal ribosome entry sites (IRES) and therefore translated in a cap-independent manner (Komar and Hatzoglou, 2005). An IRES sequence has been identified at the 5'-UTR of SNAT2 mRNA suggesting that this transporter is efficiently translated under amino acid deprivation (Fernandez et al., 2001). In addition to these mechanisms, the interaction between SNAT2 and its substrates is thought to take part in the adaptive response. Gazzola *et al.* have shown in fact that the adaptive increase in SNAT2 can be inhibited by resupply of a single substrate amino acid or the non-metabolizable substrate MeAIB (Gazzola et al., 1981). Given that resupplementation of one amino acid is not sufficient to decrease uncharged tRNA level, it follows that SNAT2 binding to a substrate induces a repressive signal that is able to block the adaptive response, although the molecular mechanisms behind this event remain unknown. One possibility is that, substrate binding induces a conformational change in SNAT2 that allows it to

initiate a signal cascade regulating its own expression. Work from the Hundal lab has identified two different pathways involved in the repression effect by single amino acids upon the adaptive response. One pathway involves JNK, which becomes activated in response to amino acid withdrawal and whose inhibition suppresses SNAT2 adaptation. The second pathway is independent of JNK but sensitive to PI3K inhibition (Hyde et al., 2007). In addition to a requirement for SNAT2 gene transcription, studies from the Hundal lab have revealed that increased SNAT2 protein stability also contributes to the adaptive response. Hyde *et al*. demonstrated that expression of a C-terminally-V5-tagged SNAT2 construct expressed under the control of a CMV promoter, which was not responsive to changes in amino acid availability, leads to increased expression and stabilisation of SNAT2-V5 protein (Hyde et al., 2007). In contrast to SNAT2, expression of a related SNAT family member, SNAT5-V5, did not result in increased stabilisation upon cellular amino-acid withdrawal. This fundamental divergence may arise because of differences in the N-terminal domain of SNAT2 and SNAT5 since a SNAT2-5 chimera, in which just the cytosolic N-terminal tail of SNAT2 was substituted for that of SNAT5, led to enhanced stabilisation of the chimeric protein in cells subjected to amino-acid withdrawal (Hyde et al., 2007). The N-terminal tail of SNAT2 harbours putative lysine ubiquitination residues and there is evidence that SNAT2 stabilization and turnover at the plasma membrane involves the ubiquitin–proteasomal pathway. Although most membrane proteins are degraded by lysosomes, some of them, such as the epithelial sodium channel, have been shown to be targeted by ubiquitin and degraded through the proteasome system (Malik et al., 2001; Malik et al., 2005). Hatanaka *et al.* have shown that SNAT2 is polyubiquitinated

and degraded by the proteasome through the action of the E3 ligase Nedd4.2 (neural precursor cell expressed, developmentally down-regulated 4-2) in 3T3-L1 adipocytes (Hatanaka et al., 2006). Data from the Hundal lab demonstrated that in L6 skeletal muscle cells treatment with the proteasome inhibitor MG132 results in increased accumulation of SNAT2. This latter observation implies that in skeletal muscle cells SNAT2 turnover at the cell membrane could be regulated by proteasomal degradation.

1.14.3 SNAT2 as a transceptor

In addition to its role as a transporter, SNAT2 is also thought to exhibit a receptor function that is capable of sensing extracellular amino acid availability and may potentially couple to the mTOR signalling pathway (Hundal and Taylor, 2009; Hyde et al., 2007). As mentioned earlier, the initial observation of Gazzola that a single substrate amino acid can repress the SNAT2 adaptive response suggested that the transporter may be able to sense extracellular amino acid availability in the extracellular space (Gazzola et al., 1981). Moreover, there is evidence that SNAT2 is capable of modulating signalling pathways. Treatment, for example, of muscle cells with a saturating concentration of the non-metabolisable substrate MeAIB enhances insulin-stimulated PI3K activity and consequent Akt phosphorylation, while silencing SNAT2 by RNA interference impairs insulin signalling through PI3K (Evans et al., 2008). Evidence of SNAT2 control of mTOR signalling has also been demonstrated in MCF-7, breast cancer cell line in which chronic exposure to MeAIB induced S6K phosphorylation (Pinilla et al., 2011). Since System A is functionally coupled to the activity of the tertiary active transporter, LAT1, it has also been implicated in the regulation of the intracellular amino-acid pool size and pathways that

influence growth and proliferation of cells. For example, the glutamine gradient generated by SNAT2 is used by LAT1 to mediate the influx of leucine, a major regulatory stimulus for the mTOR pathway and consequent regulation of processes such as growth and proliferation (Kimball et al., 1999). Bevington *et al.* have in fact shown that incubation of L6 skeletal muscle cells with MeAIB leads to decreased intracellular levels of not only SNAT2 amino acid substrates but also branched chain amino acids, whose transport is mediated by System L (Bevington et al., 2002).

1.15 Fatty acid modulation of amino acid transporter

The effect of SFAs and UFAs on insulin and stress signalling has been amply documented; however little is known about how fatty acids may affect amino acid transport. Interestingly, previous work from the Hundal lab has demonstrated that ceramide, a fatty acid derived sphingolipid, can suppress basal and insulin-stimulated SNAT2 activity by preventing its translocation to the membrane, an effect that it also exerts on GLUT4 (Hyde et al., 2005). There is mounting evidence that PUFAs affect the activity of several amino acid transporters. For example, docosahexaenoic acid (DHA) has been shown to differentially modulate the various subtypes of the glutamate transporter, Glial Glutamate Transporter 1 (GLT1), GLAST and EAAC1. DHA appears to stimulate D-[^{3}H] aspartate uptake *via* GLT1 and EAAC1 through a mechanism involving CaM kinase II and PKC; whereas the PUFA exerts an inhibitory effect on GLAST-mediated uptake in a calcium-independent manner (Berry et al., 2005). As well as DHA, arachidonic acid (ARA) can also differentially regulate members of the glutamate transporter family. ARA inhibits glutamate uptake by EAAT1 whilst increasing transport *via* EAAT2 (Zerangue et al., 1995).

1.16 Aims and objectives

Increasing evidence in the literature suggests that diets enriched with MUFAs and PUFAs can help antagonise the harmful metabolic and physiological effects associated with consuming excessive saturated fat and thereby significantly reduce the risk of developing obesity, insulin resistance and Type 2 diabetes (Bergouignan et al., 2009). However, the mechanisms by which MUFAs and PUFAs provide this protective effect against SFAs is still not fully understood. Therefore, the main aim of the studies presented in Chapter 3 was to investigate what impact different UFAs have upon insulin signalling in skeletal muscle, a primary insulin target tissue.

The specific objectives of these studies were:

- Understand how the hormonal activation of Akt and ERK1/2 is enhanced in response to linoleic and oleic acid;
- Assess whether linoleic and oleic acid can prevent the insulin-desensitising effects elicited by SFAs, such as palmitate, and define the role played by the phosphatase PP2A.

Given that, previous work from the Hundal lab has shown that ceramide, a palmitate-derived lipid, can suppress the activity of the SNAT2 amino-acid transporter (Hyde et al., 2005), a further aspect of the studies described herein was to explore the impact that SFAs and UFAs have upon SNAT2/System A transport. Investigating this topic was a major goal of the studies described in Chapter 4 whose specific aims were to:

- Assess the effect of linoleic acid, oleic acid and palmitate on expression and activity of SNAT2;

- Understand the molecular mechanisms by which linoleic acid modulates SystemA/SNAT2 activity with specific focus on the fatty acid-induced SNAT2 degradation *via* the ubiquitin proteasomal pathway.
- Assess the involvement of ceramide as a mediator of palmitate's effect upon SystemA/SNAT2 activity.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 General

α-MEM (α-minimal essential medium), DMEM (Dulbecco's modified Eagle's medium), FBS (fetal bovine serum), EBSS (Earle's Balanced Salt Solution), Opti-Mem, Trypsin/ Ethylenediaminetetraacetic acid (EDTA), penicillin/streptomycin solution, amphotericin B and Lipofectamine-2000 were from Gibco/Invitrogen Life Technologies (Paisley, UK). All other reagent-grade chemicals, including bovine serum albumin (BSA) and fatty acids, were obtained from Sigma–Aldrich (Poole, UK) unless otherwise stated. Wortmannin, PI-103, okadaic acid and polyvinylidene difluoride (PVDF) membranes were from Calbiochem-Merck Millipore (Darmstadt, Germany). Fraction V fatty acid-free BSA and complete protein phosphatase inhibitor tablets were purchased from Boehringer-Roche Diagnostics (Basel, Switzerland). Insulin was from Tocris Bioscience (Bristol, UK). Go Taq DNA polymerase, deoxyribonucleotide phosphates (dNTPs) and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase were purchased from Promega (Southampton, UK). All restriction enzymes used and alkaline calf intestinal phosphatase were from New England Biolabs (Ipswich, US). All primers were synthesised by the Oligonucleotide Synthesis Service (University of Dundee). All antibodies used in this study and their source are listed in Appendix 1. X-ray films were purchased from Konica Minolta (Tokyo, Japan).

2.2 Tissue Culture

For tissue culture, media and buffers were pre-warmed to 37°C in a water bath prior to use. Frozen cell stocks were resuscitated by heating in a 37°C water bath and then transferred into a 15 cm dish or T75 flask containing 15 ml of appropriate media for cell line propagation. To create cell stocks, cells seeded and plated from a low passage number were washed with sterile phosphate buffered-saline (PBS) (Appendix 2), trypsinised, pooled and centrifuged at 1000 *g* for 5 min. The supernatant containing the media was aspirated off and the pellet containing the cells was resuspended in storage media, which consisted of α-MEM for L6 myoblasts and DMEM for HeLa and MEFi cells. The storage media in each case was supplementend with 10% (v/v) FBS, 7% (v/v) dimethyl sulfoxide (DMSO), 1% (v/v) antibiotic/antimycotic solution (100 units/ml penicillin, 100 mg/ml streptomycin and 250 ng/ml amphotericin B). Cells were aliquoted into cryotubes and placed in a cryotube cooler container at -80°C for 24 h prior to being moved for long term storage in liquid nitrogen.

2.2.1 L6 cells

The L6 rat skeletal muscle cell line was generated originally by Yaffe from primary cultures of thigh muscle of a day old neonatal rat, maintained for the first two passages in the presence of methyl cholanthrene (Yaffe, 1968). L6 muscle cells for this study were kindly provided by Dr Amira Klip (Hospital for sick children, Toronto, Canada). L6 muscle cells were thawed from a storage stock as described above, and were cultured in α-MEM containing 2% (v/v) FBS and 1% (v/v) antibiotic/antimycotic solution (100 units/ml penicillin, 100 mg/ml streptomycin and 250 ng/ml amphotericin B) at 37°C with 5% CO_2. Cells were

splitt every 2-3 days to avoid premature differentiation. Prior to myoblast alignment, cells were washed with sterile PBS and incubated with Trypsin/EDTA for a few minutes at 37°C to detach myoblasts from the plate. α-MEM media was then added, and cells washed-off the plate and used to maintain passages or seeded onto culture plates for use in experiments. Cells of passage 2-4 were used for all experiments and seeded at a density of 4000 cells/cm^2 in appropriate dishes. Cells were maintained in culture for 7-8 days with media being replaced every 2 days, to facilitate spontaneous myoblast fusion into myotubes. Upon differentiation, cells were treated as indicated in Figure Legends.

2.2.2 HeLa cells

Hela cells were cultured in DMEM, supplemented with 10% (v/v) FBS, and 1% (v/v) antibiotic/antimycotic solution (100 units/ml penicillin, 100 mg/ml streptomycin and 250 ng/ml amphotericin B) at 37°C in an atmosphere containing 5% CO_2. After a confluent monolayer had formed, Hela cells were passaged as described previously for L6 muscle cells.

2.2.3 MEFi cells

An immortalised mouse embryonic fibroblast cell line was provided as a gift by Dr. Miguel Constancia from University of Cambridge. E14 embryos were dissected to remove liver and gonads, which cannot be used to generate MEFis, and the carcasses washed using PBS. After incubation in 1 mL 0.25% (v/v) Trypsin at 37ºC, 5% CO_2 for 30 min the digested tissues were suspended in 4 volumes of DMEM medium (DMEM, 10% (v/v) FBS, 1% (v/v) penicillin-

streptomycin, 1% (v/v) L-glutamine, 1% (v/v) MEM non essential amino acids) per tube and plated densely onto culture plates. After a 4 h incubation at 37ºC, 5% CO_2, the DMEM medium was replaced and cells maintained in culture. After 24 h in culture, cells were washed in PBS and then maintained for a further 24 h in fresh medium. The following day, cells were trypsinised and re-plated at 1:3 dilution (passage P0). MeFi cells were cultured in DMEM, supplemented with 10% (v/v) FBS, and 1% (v/v) antibiotic/antimycotic solution (100 units/ml penicillin, 100 mg/ml streptomycin and 250 ng/ml amphotericin B), at 37°C with 5% CO_2. MEFis were split and propagated as described earlier for other cell lines.

2.3 Cell stimulation

2.3.1 Fatty acid treatment

L6 myotubes were exposed to fatty acids that had been pre-conjugated for at least 1 h to 2% fatty acid-free BSA (w/v) at the concentrations and for the times indicated in Figure legends. L6 myotubes were routinely serum- deprived for the period of fatty acid exposure prior to any treatment with insulin. Serum deprivation was performed using α-MEM lacking the normal serum supplement. In some experiments, cells were incubated with inhibitors at concentrations and times indicated in Figure legends; controls were incubated with the vehicle solution lacking the inhibitor.

2.3.2 Amino acid availability experiments

For investigating the effect of fatty acids on amino acids transport, cells were incubated with EBSS supplemented with individual amino acids (2 mM). For

amino acid deprivation experiments, cells were incubated with EBSS lacking amino acids for times indicated in figure legends. Fatty acid treatment was performed as described previously.

2.4 Cell lysis

Following appropriate treatment of cells with fatty acids, insulin or inhibitors, cells were washed once with cold PBS and then lysed using appropriate volume of lysis buffer (Appendix 2). Cells were harvested from culture dishes and whole-cell lysates were centrifuged for 10 min at 3800 *g*, 4°C. The supernatant was collected and frozen in liquid nitrogen prior to being stored at -20°C.

2.5 Isolation of total cell membranes

For isolation of total membranes, cells were grown in 15 cm dishes and treated as indicated in Figure legends. Cells were washed twice with ice cold PBS and scraped off culture plates using a plastic scraper. The cell suspension was centrifuged at 700 *g* for 10 min and the resultant pellet resuspended in 3 ml of ice cold buffer I (Appendix 2). Cells were homogenized using a 5 ml Dounce glass homogenizer (40 strokes) and then centrifuged at 760 *g* for 10 min. The resulting supernatant was centrifuged at 177,000 *g* for 1 h at 4°C (Beckman TLA 100.3 rotor). The pellet obtained from this latter spin, containing the total membranes, was resuspendend in 100 µl of Buffer I supplemented with protease inhibitor and stored at -20°C.

2.6 Protein analysis

Protein concentration was measured using the Bradford method (Bradford, 1976). The assay is based on the observation that the maximum absorption of the dye Coomassie Brilliant Blue G-250, contained in the assay reagent (Biorad, Herts, UK), shifts from 465 nm to 595 nm when bound to proteins. 2 µl of standard or protein samples was added in triplicate to a 96-well plate. 200 µl of Biorad protein assay (diluted 1:5) was added to each well and the absorbance measured at 595 nm on a spectrophotometer. Bovine serum albumin (1 µg-10 µg) was used to generate a standard curve to calculate the protein concentration of experimental samples.

2.7 Immunobiology

2.7.1 Immunoprecipitation

1 mg of L6 cell lysate protein was incubated with anti-IRS1, anti-PP2Ac-demethylated or anti-V5 antibody over night at 4°C on an orbital platform shaker. Protein G–Sepharose beads were washed three times in PBS and incubated with the lysate-antibody mixture for 2 h at 4°C. Lysate-antibody/beads complex were then centrifuged at 1000 *g* for 3 min and the supernatant was removed from the beads and discarded. The immunoprecipitates were washed twice with 1 ml of lysis buffer containing 0.5 M NaCl and twice with PBS, and then boiled for 5 min in Laemmli buffer (Appendix 2) to dissociate the lysate-antibody/beads complex. The immunopellets were then resolved by Sodium Dodecyl Sulphate (SDS)-PolyAcrylamide Gel Electrophoresis (PAGE) and immunoblotted with appropriate antibodies to protein of interest.

2.7.2 SDS-PAGE and Immunoblotting

Proteins from cell lysates or total membranes were separated by SDS-PAGE as described by Laemmli (Laemmli, 1970). Whole cell lysate (30 µg) or total membranes (10 µg) were diluted in 6X Laemmli buffer and boiled for 5 min. Samples and Protein-Precision Plus molecular weight markers (New BioLabs) were resolved by running at a constant voltage (110 V) through a gel of appropriate percentage of polyacrylamide. Resolved proteins were transferred onto PVDF membrane, pre-soaked with methanol. Electrophoresis transfer was performed in cold running buffer (Appendix 1) at constant voltage of 100 V for 90 min. Proteins were visualized by Ponceau S stain and membranes blocked with 5% (w/v) milk in 0.05% (v/v) Tween 20/Tris Buffered Saline (TBST) (Appendix 2) for 1 h. Membranes were then washed once in TBST before incubation overnight with primary antibodies (as indicated in Figure legends). Primary antibody was removed and PVDF membranes were washed three times for 15 min in TBST. Membranes were then incubated with an appropriate peroxidase-conjugated IgG for 1 h at room temperature and then washed three times for 15 min in TBST. Immunoreactive bands were detected by enhanced chemiluminescence (Appendix 2) on Kodak X-OMAT film. Quantification of immunoblots was performed using Image J software (http://rsbweb.nih.gov/ij/).

2.8 Measurement of phosphatidylinositol 3,4,5 trisphosphate (PI(3,4,5)P_3)

The effects of fatty acids and insulin on cellular PI(3,4,5)P_3 content was assessed using a sensitive time-resolved FRET (fluorescence resonance energy transfer)-based assay. This assay monitors the displacement of GST-tagged GRP1 (general receptor of phosphoinositides 1)-PH domain from a

sensor complex consisting of Eu Lance® chelate-labeled GST antibody, the GST-tagged GRP1-PH domain, biotinylated-PI(3,4,5)P_3 and the FRET acceptor, streptavidin allophycocyanin, by nonbiotinylated lipid (Gray et al. 2008). After the appropriate incubation of cells with insulin or the PI3K inhibitor PI-103, cells were rapidly washed and cellular material precipitated by the immediate addition of 0.5 ml of ice-cold 0.5 M trichloroacetic acid (TCA). After standing on ice for 5 min, cells were harvested from the plates and the acid precipitate pelleted by centrifugation. The pellet was washed two times with 1 ml of 5% TCA, 1 mM EDTA. Neutral lipids were extracted from the pellet and PI(3,4,5)P_3 content determined as described previously using an LJL Analyst plate reader (Gray et al, 2008). PI(3,4,5)P_3 abundance was calculated by reference to a standard curve constructed by addition of known amounts of the 3-phosphoinositide to the sensor complex.

2.9 Amino acid uptake

Cells, seeded in 12 well plates, were washed twice with Hepes buffered saline (HBS) (Appendix 2) prior to incubation with uptake buffer (HBS, 10 μM MeAIB and 0.0074MBq [^{14}C]-MeAIB tracer, or 10 μM leucine and [^{14}C]-Leucine tracer). After the assay period (10 min for MeAIB and 2 min for Leucine uptake) the uptake was terminated by rapidly aspirating the media and washing cells twice with cold 0.9% (w/v) NaCl. Cells were lysed in 50 mM NaOH and 1 ml of the lysate was transferred into 6 ml scintillation vials with addition of 4 ml of scintillation fluid (Ecoscint, national Dagnostics). Cell radioactivity was measured using a 1450 microbeta liquid scintillation counter. Protein concentration was determined from the rest of the lysates using the Bradford

method as described before. Uptake of radiolabelled substrate was calculated using the equation as follow:

$$\text{Flux} = \frac{(RAD_{spec})-(RAD_{quench})}{RAD_{sa} * P * T}$$

(units= pmol/min/mg/protein)

RAD_{spec} = measured radioactivity associated with 1 ml of cell lysate (disintegrations per min (DPM)/ml)

RAD_{quench} = measured radioactivity associated with 1 ml of cell lysate in the presence of saturating dose of substrate (DPM/ml)

RAD_{sa} = measured radioactivity associated with 1 pmol of transport substrate (specific acitivty, DPM/ml)

P = protein concentration of cell lysate (mg/ml)

T = time span of assay (min)

2.10 shRNA mediated silencing of AMPK, Caveolin and Nedd4.2.

Expression of Ampk α1, Caveolin1/3 and Nedd4.2 was silenced in L6 myoblasts using a lentiviral knockdown strategy. Knockdown of the above proteins was performed by Dr Clare Stretton.

2.10.1 Hairpin design

Short hairpin interfering RNA (shRNA) sequences targeting the genes of interest were found on Sigma-Aldrich website. To generate oligos for cloning,

the selected shRNAs were inserted into a replication-incompetent lentiviral vector pLKO.1. Each shRNA sequence was inserted into the two oligo sequences below (Appendix 3).

Forward oligo:

5’ CCGG – 21bp sense – CTCGAG – 21bp antisense – TTTTTG 3’

Reverse oligo:

5’ AATTCAAAAA – 21bp sense – CTCGAG – 21bp antisense 3’.

2.10.2 Cloning oligos in PLKO.1

To allow the annealing of forward and reverse oligos, 2 µg of each were added to 2 mM Tris-HCl (pH 8.5) in a total volume of 50 µl, heated to 94°C for 12 min followed by cooling by 1°C each minute until 21°C. 6 µl of 10x ligation buffer, 3 µl of water and 1 µl of T4 polynucleotide kinase were added and the mixture incubated at 37°C for 30 min, followed by incubation at 70°C for 20 min to inactivate the enzyme. 10 µg of pLKO.1 vector was digested with 2 µl EcoRI and 2 µl AgeI for 2 h at 37°C and then incubated for 1 h at 37°C with calf intestinal phosphatase. For the ligation reactions 100 ng of plasmid was added to 2 µl of the annealed oligo mixture, 2U of T4 DNA ligase and an appropriate amount of ligase buffer and water for a total volume of 10 µl followed by incubation at room temperature for 2-3 h. 5 µl of ligation reaction was transformed into 50 µl of XL-1 cells using a standard heat shock method and half of the transformation was plated out onto ampicillin plates and incubated overnight. Colonies were picked and screened using primers to pLKO.1 (see Appendix 4 for sequences) and run on a 1.5% agarose gel, using pLKO.1

containing a scrambled shRNA sequence insert as a positive control. To confirm the insertion of hairpin sequences into the pLKO.1 vector, 1 µg of each plasmid (including the empty vector and one containing the scrambled sequence) was digested with AgeI, a site which is lost after insertion of hairpin oligos and the products were run out on a 0.8% agarose gel. The plasmids lacking the AgeI site were identified and sequenced using the reverse primer to confirm the presence of the shRNA.

2.10.3 Preparation of Lentivirus

HEK 293T cells were seeded onto 6 cm dishes (one for each hairpin, plus one each for the empty vector and the scramble vector) one day prior to transfection at a density of 1.5 x 10^6 cells per dish. For each hairpin a DNA mixture for the transfection, consisting of 1.5 µg of pLKO.1, 1 µg of pHR CMV8.2ΔR packaging and 1 µg pCMV VSVg envelope vector was added to 0.5 ml serum-free medium. 12.5 µl 1 mg/ml Polyethylenimine was added to the solution, mixed and incubated at room temperature for 30 min. Cell media was replaced with fresh 2.5 ml complete medium and the transfection mix was added to cell culture plate for incubation overnight. From this point on cells were considered virally infectious. Medium was replaced the day after with 3 ml of complete medium and incubated for a further 48 h. The media containing the released virus was collected, centrifuged for 5 min at 200 *g* to pellet any residual cells, then divided into 1 ml aliquots and stored at -80°C.

2.10.4 Lentiviral infection of L6 and HeLa cells

L6 muscle cells or HeLa cells were seeded into 6 cm dishes one for each hairpin plus a control with approximately 20,000 cells and incubated for 4-6 h. 1

ml of virus was mixed with 2 ml of complete medium and polybrene was added to a final concentration of 8 µg/ml. The media on cells was replaced with this mix and cells were considered to be virally infectious from this point on. After 24 h of incubation the media on cells was replaced with 3 ml medium containing 3 µg/ml puromycin (for cells in which AMPK α1 and Nedd4.2 were silenced) or 15 µg/ml hygromycin (for the Caveolin1/3 knock down cell line). The pLKO.1 vector encodes gene which confers either puromycin or hygromycin resistance. Consequently cells that have incorporated pLKO.1 into the host genome will be resistant to antibiotic treatment. Media was replaced with fresh antibiotic-containing media every two days until all cells in the control plate were dead. After five media changes, cells were no longer considered as infectious. Once a sufficient number of cells had grown, the established cell lines were screened by western blot for decreased levels of target protein expression to determine which shRNAs were successful. Cell lines expressing the empty vector and scrambled sequence were used as positive control.

2.11 Molecular biology

2.11.1 RNA extraction

Total RNA was extracted from L6 myotubes using TRIzol® reagent according to the manufacturer's instructions (Sigma–Aldrich). Cells were grown in 6 cm dishes and following treatments were washed in ice cold PBS and then scraped and collected in TRI reagent. 0.2 ml of chloroform per ml of TRI reagent used was added. Samples were shaken and, after 5 min incubation at room temperature, were centrifuged at 18,000 *g* for 15 min at 4°C. The upper aqueous phase containing RNA was transferred into a new 1.5 ml tube and 0.5

ml of 99.9% isopropanol per ml of TRI reagent added. Samples were shaken and after 10 min incubation at room temperature, centrifuged at 18,000 *g* for 10 min at 4°C. The supernatant was removed and the pellet containing RNA was washed in 1 ml 70% (v/v) ethanol per ml of TRI reagent and centrifuged at 12,800 *g* for 5 min at 4°C. The ethanol was removed and the pellet air-dried and resuspended in 15 µl of DEPC water. After 10 min incubation on ice, RNA samples were quantified using an Ultraspec 2100 pro UV/Visible Spectrophotometer (Amersham Biosciences) and then stored at -80°C.

2.11.2 Generation of cDNA

Complementary DNA was generated from 1 µg of RNA. RNA was added to 0.5 µg of oligo T and DEPC water up to a volume of 17.7 µl and incubated at 70°C for 5 min to denature the secondary structure. Samples were then cooled to allow oligo T annealing to the poly A tail of messenger RNA. The reaction mix composed of 5 µl M-MLV reverse transcriptase 5X reaction buffer, 1.3 µl 10 mM dNTP, 1 µl M-MLV reverse transcriptase was added and the samples incubated at 42°C for 1 h to allow reverse transcription. After this period the mixture was incubated for 10 min at 70°C to inactivate the enzyme. Samples were stored at -20°C.

2.11.3 Real-time quantitative-polymerase Chain Reaction

Quantitative real-time PCR was carried out using a StepOnePlus Real-Time PCR System (Applied Biosystems), SYBR Green JumpStart Taq ReadyMix (Sigma–Aldrich) and primers targeting the gene of interest. 3 µl of cDNA diluted 1:15 in Milli-Q water were added to 5 µl of SYBR Green, 1 µl of forward (2 µM)

and 1 µl of reverse (2 µM) primers. The sequences for these primers are shown in Appendix 4. PCR amplification was performed with an initial denaturation at 95 C for 10 min followed by 40 cycles of denaturation at 95 C for 15 s, annealing at 55°C for 15 s and extension at 68°C for 1 min. The ratio of target gene mRNA expression to housekeeping gene mRNA expression were calculated as described previously (Pfaffl et al, 2001).

2.11.4 Plasmid constructs

pCDNA6-SNAT2-V5 was generated by Dr Russel Hyde, by introducing SNAT2 into a tagging vector so that a simian virus 5 hexahistidin epitope (V5-His6) could be introduced at the carboxyl-terminus of the protein. Rat SNAT2 was PCR amplified using the primers shown in Appendix 4 and introduced into pcDNA6 V5-His6 version A (Invitrogen) using XhoI and XbaI.

pCDNA6-7A-SNAT2 mutant was generated by Dr Thorsten Hoffmann. The SNAT2 DNA sequence in which the 7 lysines in the N-terminus have been mutated to alanine was generated by Life Technologies. SNAT2-7A sequence was then introduced into pcDNA6 V5-His6 version A (Invitrogen) using XhoI and XbaI.

pCMV5-Ubiquitin-FLAG was obtained from Division of Signal Transduction Therapy at the University of Dundee.

2.11.5 Transformations

All DNA plasmids were transformed into XL1-Blue competent cells *E.coli*. 1 µl of plasmid was added to 50 µl of *E.coli* cells and incubated on ice for 20 min. The mixed solution was then heat-shocked at 42°C for 1 min and put back on ice.

250 µl of Luria-Bertani (LB) media lacking of antibiotic was added to the solution followed by incubation at 37°C on a shaking incubator for 1 h. Half of the solution was then plated onto appropriate antibiotic containing LB agar plates and incubated overnight at 37°C.

2.11.6 Plasmid purification

Single colonies were picked from the LB-agar plates and grown in 5 ml of LB media containing the appropriate antibiotic. The cells were grown at 37°C in a shaking incubator for 8-15 h. Cells were then either pelleted for plasmid preparation (miniprep) or transferred to the media (200 ml for maxi prep) containing antibiotic and incubated overnight in 37°C in a shaking incubator. Mini-prep and Maxi-prep were performed according to the manufacturer's protocols (Qiagen).

2.11.7 Transient transfection

HeLa cells were transfected with pCDNA6-V5 or pCMV5-FLAG constructs by using either Lipofectamine or polyethilenimine (PEI) as indicated in Figure legends.

In the first case, cells were seeded in 6 cm dishes until 70% confluent for transfection. For each dish a solution containing 250 µl of Opti-Mem plus 10 µl of Lipofectamine-2000 and one containing 250 µl of Opti-Mem plus 5 µg of DNA were prepared and incubated at room temperature in a tissue culture hood for 5 min. The solution containing Lipofectamine was then added to the DNA and mixed by tapping the tube. After a 20 min incubation at room temperature the mixed solution was added drop wise into culture media of a 6 cm dish of HeLa

cells. Cells were incubated for 24 h at 37°C before being treated as described in section 2.3.

For transfection with the PEI method HeLa cells were seeded in 10 cm dishes until 70% confluent. 15 µl of PEI were added to a solution of serum-free DMEM containing 10 µg DNA vector and immediately vortexed for 5-10 s. The solution was incubated at room temperature in a tissue culture hood for 15-30 min before being added drop wise into culture media of a 10 cm dish of HeLa cells. Cells were incubated for 24 h at 37°C and then splitted into experimental culture plates. 48 h post-transfection cells were treated as described in section 2.3.

2.12 Statistical analyses

For multiple comparisons, statistical analysis was performed using one-way ANOVA. For individual comparisons statistical analysis was performed using a Student's *t* test. Data analysis was performed using GraphPad Prism software and considered statistically significant at $P<0.05$.

Chapter 3

Unsaturated fatty acid provision enhances insulin sensitivity and ameliorates palmitate-induced insulin resistance in skeletal muscle cells

3.1 Introduction

Sustained increases in the circulating concentration of free fatty acids (FFA), as often occurs in plasma of obese or type 2 diabetic individuals, have been linked to impaired insulin-dependent control of blood glucose (Boden, 2011; Kahn et al., 2006). In particular, saturated fatty acids (SFA) have been implicated strongly in the pathogenesis of insulin resistance in skeletal muscle, a tissue, which, by virtue of its sizable contribution to lean body mass, represents a major site for the disposal and metabolism of circulating glucose in the postprandial state (DeFronzo et al., 1985). Numerous studies have shown that chronic over-provision of SFAs (*e.g.* palmitate) promote a significant reduction in the insulin signalling capacity of skeletal muscle which may help account for disturbances in whole body glucose metabolism in individuals who are obese or diabetic (DeFronzo et al., 1985). Several studies have demonstrated that supply of SFAs in excess of what skeletal muscle can safely utilise lead to the tissue accumulation of lipotoxic fatty acid derivatives, such as DAG, ceramide and gangliosides like GM3, which impact negatively upon insulin action. Increases in DAG induce activation of DAG-sensitive PKCs that promote serine phosphorylation of IRS proteins thereby reducing their insulin signalling potential. Intramyocellular accumulation of ceramide on the other hand disrupts Akt-directed insulin signalling by mechanisms that involve activation of atypical PKCs and PP2A, which retain Akt in a repressed and dephosphorylated state (Cazzolli et al., 2001; Galbo et al., 2011; Powell et al., 2003; Powell et al., 2004; Stratford et al., 2004). The ganglioside GM3 impairs insulin signalling by a mechanism involving the dissociation of insulin receptors from caveolin-1, thus uncoupling them from their downstream signalling effectors (Inokuchi, 2009). In

addition to the lipotoxic effects of DAG and ceramide, palmitate (PA) also invokes an increase in NFkB-dependent proinflammatory signalling and an ER stress response (Green et al., 2011; Hage Hassan et al., 2012). Precisely how these latter responses are triggered by PA availability and whether they play a role in the development of insulin resistance is currently unclear given that their pharmacological repression fails to negate the PA-induced loss in insulin sensitivity (Green et al., 2011; Hage Hassan et al., 2012). In addition to promoting a rise in intramyocellular ceramide and DAG, SFAs also affect cellular energy balance. Indeed, there is mounting evidence suggesting that many of the metabolic defects in muscle associated with sustained PA oversupply may arise through an inability to match respiratory drive with fuel supply and that the heavy metabolic burden ultimately results in mitochondrial dysfunction, cell stress and insulin resistance (Koves et al., 2008; Lipina et al., 2013).

Strikingly, monounsaturated fatty acids (MUFAs) or certain polyunsaturated fatty acids (PUFAs) do not initiate some of the adverse metabolic responses associated with oversupply of SFAs. Indeed, evidence in the literature indicates that dietary substitution of SFAs or co-supplementation with unsaturated fatty acids antagonise the insulin desensitising effects of SFAs, enhance energy expenditure and improve muscle lipid composition and serum acylcarnitine profiles in humans (Fedor and Kelley, 2009; Gillingham et al., 2011; Kien et al., 2013b; Kien et al., 2011; Parillo et al., 1992). Such observations are in line with cell-based studies demonstrating that MUFAs, such as palmitoleate and oleic acid, prevent the suppressive effect of PA on mitochondrial biogenesis and function *via* a peroxisome proliferator-activated receptor alpha dependent

mechanism (Yuzefovych et al., 2010), and antagonise cellular inflammation and ER stress by an AMPK-mediated mechanism (Salvado et al., 2013; Xue et al., 2012). Moreover, the observation that specific enrichment of serum with palmitoleate induces a strong insulin potentiating effect in mouse liver and skeletal muscle implies that this MUFA may possesses unique metabolic attributes not held by its saturated counter-part, PA (Cao et al., 2008). However, despite the observed gain in insulin sensitivity brought about by palmitoleate in these peripheral tissues, our understanding of the underlying mechanism remains poor (Cao et al., 2008).

In this chapter I investigated the effects of two of the most common unsaturated fatty acids (UFAs), oleic acid (OA) (a cis-C18:1, n-9 MUFA) and linoleic acid (LOA) (a cis-C18:2, n-6 PUFA), upon insulin signalling events in cultured rat and human skeletal myotubes. I have found that both OA and LOA induce an increase in insulin sensitivity based on enhanced Akt- and ERK1/2-directed insulin signalling. These effects cannot be attributed to an elevation in upstream IRS1/PI3K signalling, suggesting that modulation of a phosphatase may be involved in the regulation of both Akt and ERK1/2. In this respect, the insulin sensitivity promoted by the unsaturated fatty acids was associated with increased tyrosine phosphorylation (Y307) and demethylation of the phosphatase PP2A – events thought to retain the phosphatase in a repressed state (Janssens and Goris, 2001; Seshacharyulu et al., 2013). In contrast, PA attenuated the tyrosine phosphorylation and demethylation of PP2A induced by insulin in line with the known stimulatory effects that this SFA has upon PP2A activity. These PA-induced changes in PP2A were potently repressed by both

OA and LOA. My data indicate that increases in insulin sensitivity brought about by exposure of muscle cells to OA and LOA may, in part, be mediated by covalent modification and cellular repression of PP2A.

I also investigated whether the beneficial effect of OA and LOA provision may involve changes in fatty acid β-oxidation, given that insulin resistance caused by PA has been linked to its reduced oxidation and greater partitioning into lipotoxic PA-derivatives (Macrae et al., 2013). However, even in presence of a CPT-1 inhibitor, OA and LOA still exert their beneficial effect. In the end, I found that the sensitising effect does not involve key protein of insulin signalling and energy metabolism, such as caveolin1/3 and AMPK, as resulted by using skeletal muscle cell line knock down for these proteins.

The aims of the studies reported in this chapter were as follows:

- To investigate the effects of OA and LOA upon insulin signalling in skeletal muscle cells.
- To investigate how OA and LOA may confer protection against PA-induced insulin resistance.
- To assess whether the insulin-sensitising effect associated with provision of OA and LOA may also be supported by modulation of AMPK and caveolins 1 and 3, which have been implicated in the control of cellular energy balance and metabolism.

3.2 Results

3.2.1 Effects of different unsaturated fatty acids on insulin signalling

The effect of different unsaturated fatty acids upon insulin signalling was investigated using a sub-maximal (20 nM) insulin concentration, determined using Akt^{S473} phosphorylation as a readout for proximal insulin signalling. At this concentration, insulin induced a modest increase in Akt phosphorylation, which was barely detectable in untreated cells (Fig 3.1A). L6 myotubes were incubated with serum free medium in the absence or presence of 700 µM elaidic acid (EA, trans-C18:1, n-9), oleic acid (OA, cis-C18:1, n-9), α-linolenic acid (α-LNA, cis-C18:3, n-3), γ-linolenic acid (γ-LNA, cis-C18:3, n-6) and linoleic acid (LOA, cis-C18:2, n-6) for 16 h, prior to insulin stimulation for the penultimate 10 min. The fatty acid concentration used in these studies was similar to the total free fatty acid concentration found in serum of obese individuals and widely regarded as pathophysiological. Cell lysates were then analysed by immunoblotting to determine the level of phosphorylation of Akt^{S473} (Fig 3.1B) and that of $ERK1/2^{T202/Y204}$ (Fig 3.1C), which are also activated in response to insulin stimulation in L6 cells. The results show that the insulin-induced phosphorylation/activation of Akt and ERK1/2 was significantly increased by exposure to oleic acid, α-linolenic acid and linoleic acid. In contrast, γ-linolenic acid exerted an inhibitory effect on both kinases whilst elaidic acid significantly enhanced ERK1/2 phosphorylation, but did not have any apparent effect upon Akt activation. Since oleic acid (OA) (a MUFA) and linoleic acid (LOA) (a PUFA) induced the greatest insulin-sensitising effect with respect to Akt activation, I focussed on these two specific fatty acids.

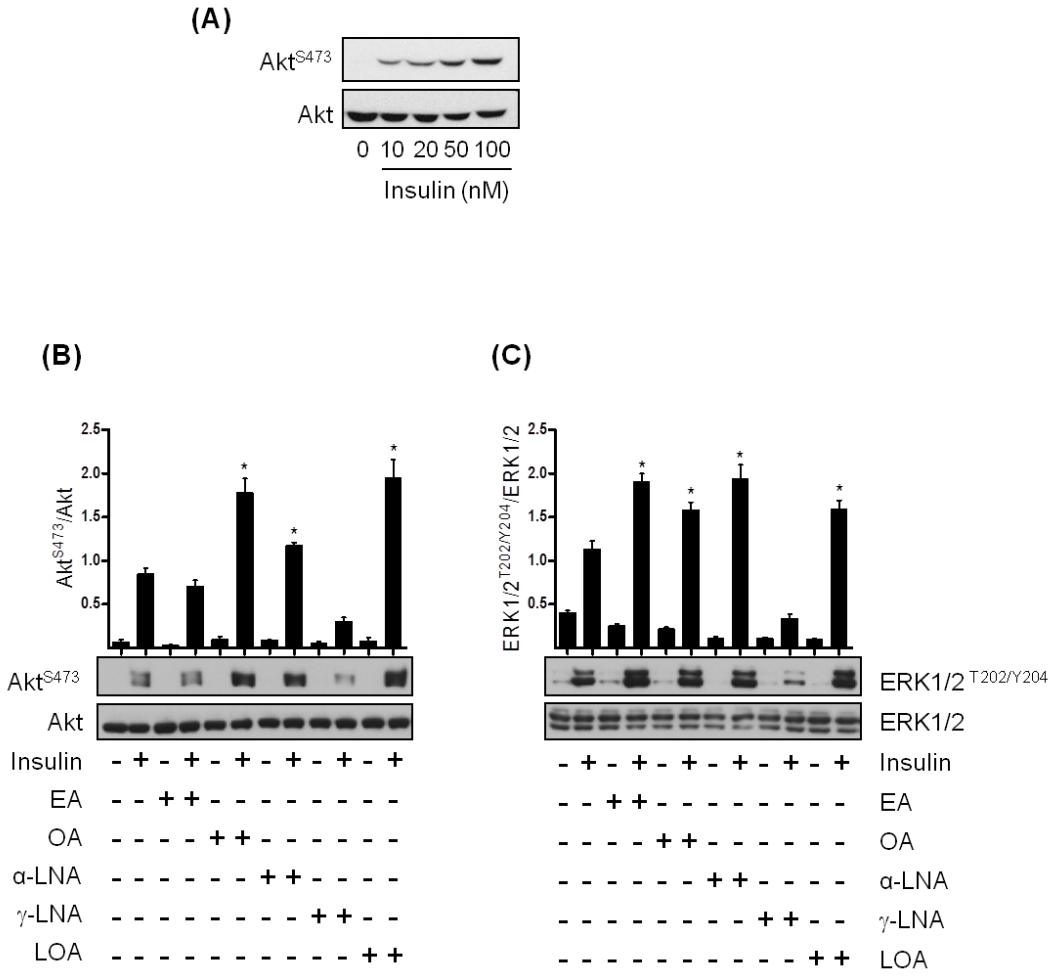


Figure 3.1 Effect of different 18C unsaturated fatty acids on Akt and ERK phosphorylation in L6 myotubes.

(A) L6 myotubes were serum-starved for 2 h prior to stimulation with insulin (10 min) at concentrations indicated. **(B, C)** L6 myotubes were incubated with serum free medium containing 2% (w/v) BSA (vehicle) ± 700 μM elaidic (EA), oleic (OA), α-linolenic (α-LNA), γ-linolenic (γ-LNA) or linoleic (LOA) acid for 16 h, prior to stimulation with or without insulin (20 nM) for 10 min. Cell lysates were analysed by immunoblotting for phospho Akt (S473) and Akt (A, B), or for phospho ERK1/2 (T202/Y204) and ERK1/2 (C). Values in the bar graphs represent the mean ± SEM from three separate experiments. Asterisks represent a significant change from the insulin-treated value alone ($P<0.05$).

3.2.2 Effects of OA and LOA on Akt and ERK1/2 activation

In order to analyse any potential effect of OA and LOA acid upon insulin signalling a time and dose-response study was performed for both the unsaturated fatty acids. Akt phosphorylation was enhanced significantly when myotubes were preincubated with OA or LOA for 16 h in a dose-dependent manner. Concentrations as low as 200 μM (*i.e.* within the physiological range) were able to cause an enhancement in Akt phosphorylation, which was ~2-fold greater than that elicited by insulin treatment alone when myotubes were pre-exposed to maximally effective doses (700 μM) of OA or LOA (Fig 3.2A and B). The increase in Akt phosphorylation was detectable after 9 h but was not maximal until 16 h of fatty acid treatment (Fig 3.2C and D). Fig 3.2C and 3.2D show that the increase in signalling was not restricted to Akt and that ERK1/2 phosphorylation was also enhanced. In the absence of insulin, neither fatty acid had any effect on Akt or ERK1/2 phosphorylation (Fig 3.2C and D). It is also important to stress that treatment of myotubes with the vehicle (*i.e.* BSA) alone did not enhance insulin-dependent phosphorylation of Akt or ERK when compared with cells not exposed to the vehicle (Fig 3.3).

3.2.3 OA and LOA improve insulin sensitivity in both rat and human muscle cells without affecting proteins lying upstream Akt

The above studies utilised a sub-maximal insulin dose to amplify any potential insulin-sensitising effect of the fatty acids, but, in parallel, I also compared the effect of both fatty acids in cells treated with sub-maximal and maximally effective insulin doses. Fig 3.4A and B show that the increased sensitisation towards insulin elicited by both NEFAs was most apparent and significant when

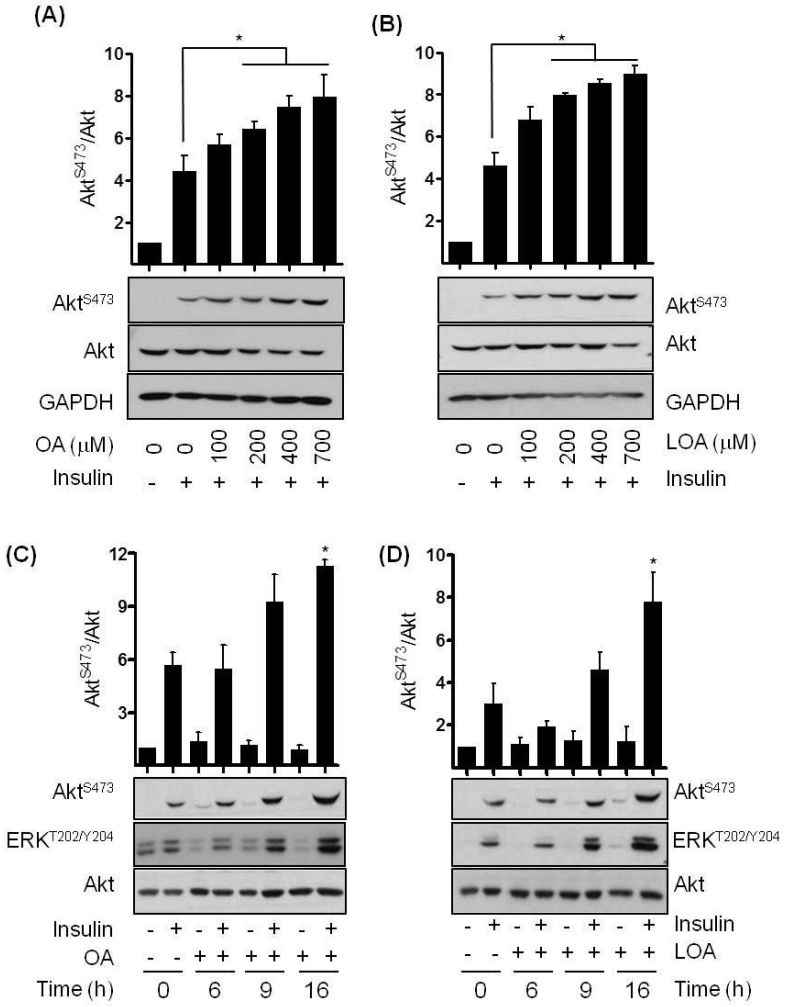


Figure 3.2 Effects of OA and LOA on Akt and ERK phosphorylation in L6 myotubes.

(A,B) L6 myotubes were incubated with serum-free media containing 2% (w/v) BSA (vehicle) ± (A) oleic acid (OA) or (B) linoleic acid (LOA) at concentrations indicated, for 16 h prior to stimulation with insulin (20 nM, 10 min). **(C, D)** L6 myotubes were incubated as in (B) ± 700 µM (C) OA or (D) LOA for times indicated, prior to stimulation with insulin (20 nM, 10 min). (A-D) Cell lysates were immunoblotted using antibodies against the proteins indicated. The asterisks signify a significant change between the indicated bars (A, B) or compared to the insulin-treated value alone (C, D) ($P<0.05$).

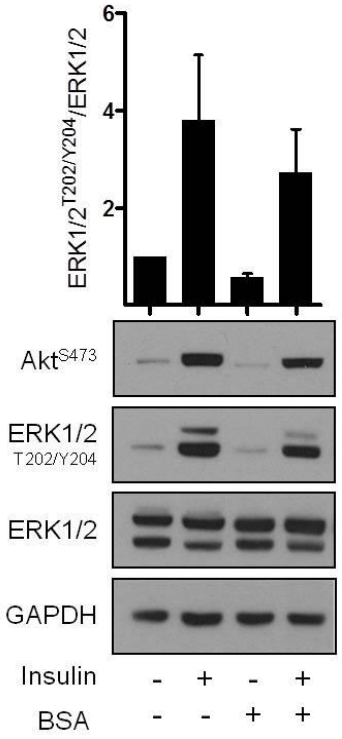


Figure 3.3 BSA does not affect insulin signalling activation in L6 myotubes.

L6 myotubes were incubated with serum-free media ± 2% (w/v) BSA for 16 h prior to stimulation with insulin (20 nM, 10 min). Data in the bar graphs is presented as mean + SEM (n=3).

monitored using a sub-maximal insulin dose as no additional increase in Akt phosphorylation was observed when OA- and LOA-treated myotubes were treated with a maximally effective (100 nM) insulin concentration. Since the sensitising effect was most apparent after a chronic fatty acid treatment (9 h), I wanted to assess the possibility that OA and LOA may enhance insulin signalling by increasing the expression and/or activation of key proteins implicated in insulin pathway. Fig 3.4C shows that there was no major change in the expression of the insulin receptor β-subunit, IRS-1, PI3K-p85 subunit or PTEN, all of which lie upstream of Akt and ERK1/2, in response to OA or LOA treatment. Furthermore, neither OA nor LOA had any effect on total Akt or ERK1/2 abundance thus excluding this as a possible explanation for the net increase in their phosphorylation. The data highlights that in addition to promoting Akt^{S473} phosphorylation, both OA and LOA induce an equivalent gain in Akt^{Thr308} phosphorylation (Fig 3.4C and D). Equimolar phosphorylation of both sites is required for full activation of Akt and consistent with this I observed elevated phosphorylation of GSK3; a physiological downstream Akt target. Likewise, enhanced ERK1/2 activation promotes greater phosphorylation/activation of CREB, a transcription factor regulated downstream of the ERK pathway (Fig 3.4C). The increase in insulin-stimulated Akt phosphorylation induced by OA and LOA was not restricted to L6 myotubes. Fig 3.5 shows that incubation of human skeletal myotubes with OA or LOA caused a similar enhancement in Akt and ERK1/2 phosphorylation in response to insulin and that of GSK3 and CREB, which respectively lie downstream of Akt and ERK signalling.

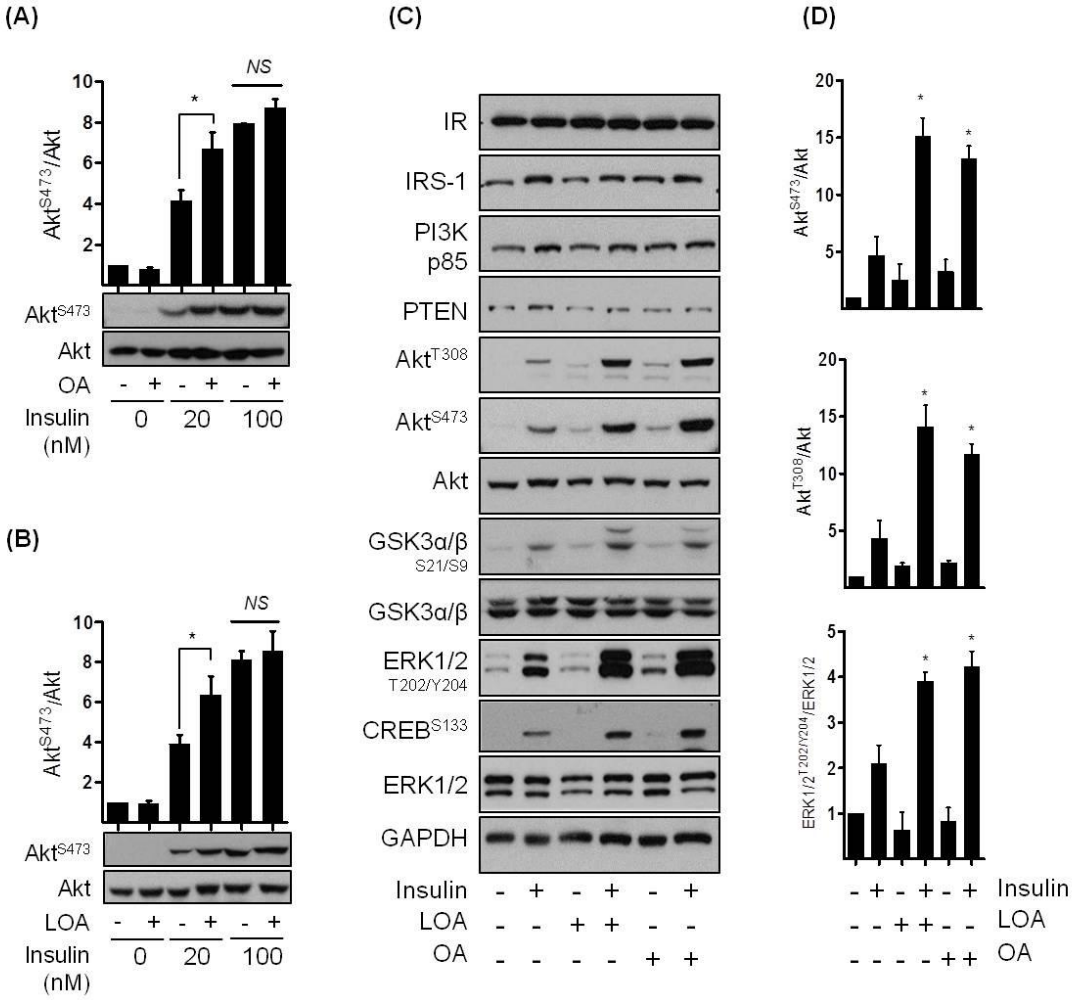


Figure 3.4 OA and LOA effect upon insulin signalling in L6 myotubes.

(A, B) L6 myotubes were incubated with serum-free media containing 2% (w/v) BSA ± 700 µM (A) oleic acid (OA) or (B) linoleic acid (LOA) for 16 h prior to stimulation with insulin at concentrations indicated (10 min). **(C, D)** L6 myotubes were incubated as in (A, B) prior to stimulation with insulin (20 nM, 10 min). Cell lysates from A-C were subsequently immunoblotted using antibodies against the proteins indicated with quantification of phospho-Akt (S473 and T308) and phospho-ERK1/2 shown in panel A, B and D expressed as mean ± SEM of at least three experiments. The asterisks signify a significant change ($P<0.05$) between the indicated bars (A, B) or the insulin-treatment alone (D).

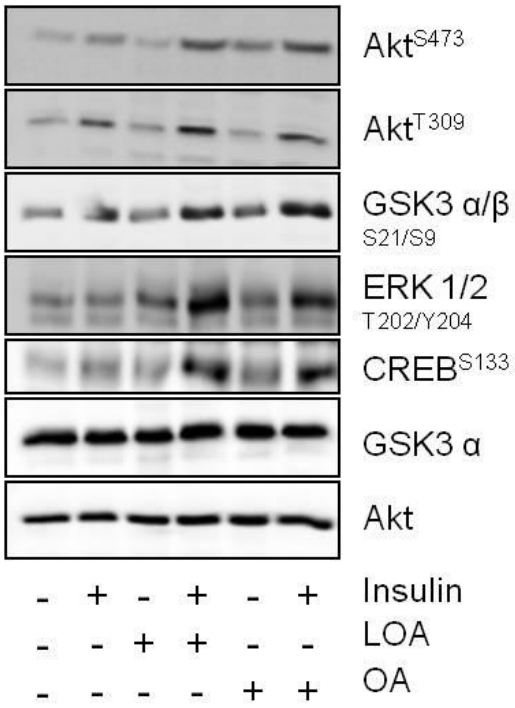


Figure 3.5 OA and LOA improve insulin sensitivity in human myotubes.

Human myotubes were incubated with 200 µM oleic acid (OA) or linoleic acid (LOA) for 24 h prior to stimulation with insulin (20 nM, 10 min). Cell lysates were subsequently immunoblotted using antibodies against the proteins indicated. The immunoblots shown are representative of two separate experiments.

3.2.4 OA and LOA sensitise muscle cells to insulin but not nutrients

Since insulin and nutrient-sensing *via* the mTOR pathway are closely linked, I investigated whether the effect elicited by OA and LOA could enhance sensitivity of the mTOR pathway to nutrient (amino acid) stimulation. To check this possibility, L6 myotubes were incubated with serum free (Fig 3.6A) or serum containing (Fig 3.6B) medium in the absence or presence of 700 µM OA or LOA. L6 cells were then stimulated with 20 nM insulin for 10 min (Fig 3.6A) or with amino acids for 20 min, after 2 h of starvation (Fig 3.6B). As expected, only the rise in insulin modulated Akt, GSK3 and ERK1/2 activation, whereas amino acid stimulation did not activate the Akt-GSK3 axis or MAPK pathway; however, LOA treatment increased ERK phosphorylation in starved cells independently of amino acid replacement (Fig 3.6B). In addition, the fatty acid treatment did not elicit any sensitising effect upon activation of P70S6K, a downstream mTOR target, both after insulin and amino acid stimulation.

3.2.5 OA and LOA insulin sensitising effect is not due to a hyperactivation of IRS1/PI3K axis

To understand how OA and LOA might enhance insulin-signalling I monitored the effect of both fatty acids on p85-PI3-kinase association with IRS1 and cellular PI(3,4,5)P_3 synthesis. As anticipated, based on coprecipitation analysis, Fig 3.7A shows that insulin induced p85-PI3K interaction with IRS1. This interaction was not influenced by OA or LOA. Since Akt activation relies upon insulin-dependent PI(3,4,5)P_3 synthesis it is possible that OA and LOA may have enhanced production of this lipid. However, Fig 3.7B shows that, irrespective of whether muscle cells were incubated in the absence or presence

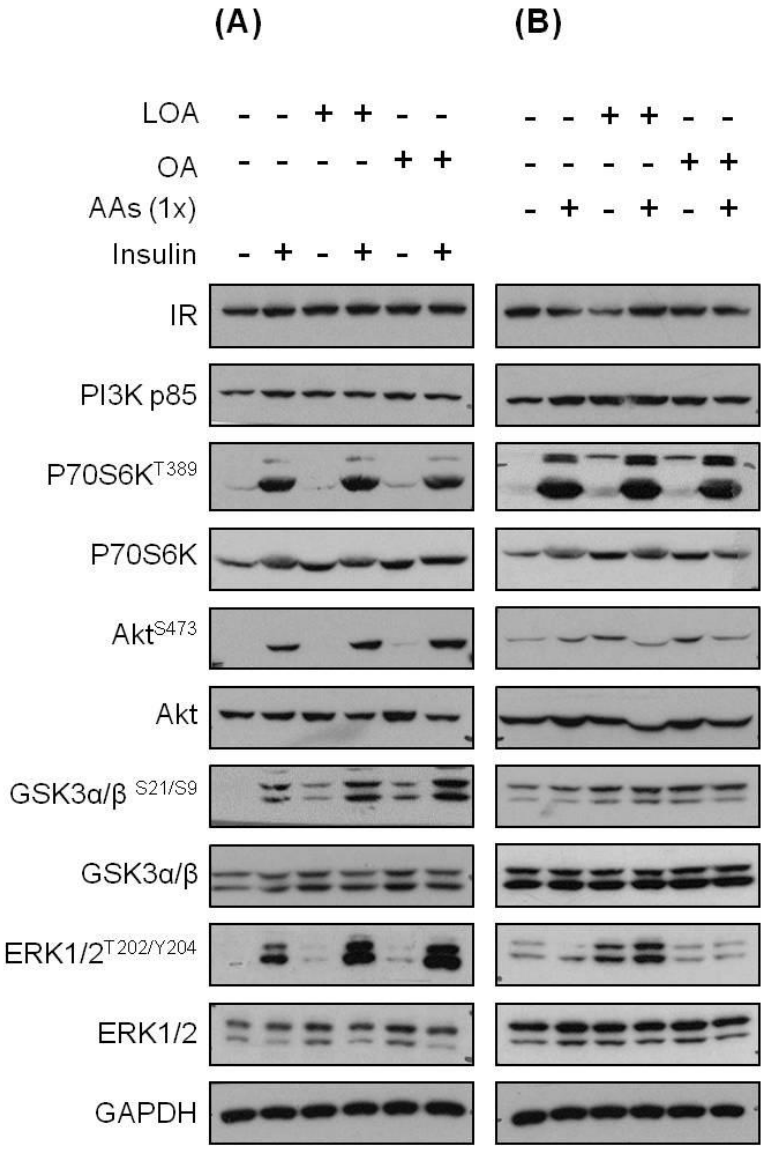


Figure 3.6 Unsaturated fatty acids appear to only sensitise cells to insulin and not nutrients in L6 myotubes.

(A, B) L6 myotubes were incubated with serum free (A) or serum-containing medium (B) containing 2% (w/v) BSA (vehicle) in the absence or presence of linoleic (LOA) or oleic acid (OA) (700 μM) for 16 h. Cells were stimulated in the absence or presence of insulin (20 nM) for 10 min (A) or amino acid for 20 min, after being starved in the penultimate 2 h of 16 h of treatment (B). Immunoblots are representative of three separate experiments.

of OA or LOA, insulin stimulated PI(3,4,5)P_3 synthesis by ~2-fold.

3.2.6 OA and LOA help sustain Akt and ERK1/2 activation

Given that signalling events at the level of IRS1/PI3K were unaffected by OA or LOA, the possibility that increased phosphorylation/activation of Akt and ERK1/2 by insulin may be a consequence of their reduced dephosphorylation was investigated. L6 myotubes were incubated in the absence or presence of these fatty acids for 16 h and subject to an acute insulin challenge in the penultimate 10 min period of this fatty acid incubation. Myotubes were subsequently washed to remove extracellular insulin and reincubated in media with 100 nM wortmannin (to inhibit PI3K and any PI(3,4,5)P_3 synthesis still occurring following removal of insulin) for up to 60 min post-wash. Muscle cells were lysed at time points indicated in Fig 3.8A and Akt and ERK1/2 phosphorylation assessed in cell lysates. In the absence of OA or LOA treatment, Akt and ERK1 were rapidly dephosphorylated upon removal of insulin and returned to baseline by between 15-30 min. However, both kinases retained a greater level of phosphorylation (~2-fold at each post-wash time point) in myotubes pretreated with OA or LOA (Fig 3.8B-E). Analysis of the $t_{1/2}$ with which Akt was dephosphorylated revealed that this was increased from 13 min (untreated cells) to 18 min and 25 min in cells that had been preincubated with OA or LOA, respectively. Similarly, the $t_{1/2}$ with which ERK1/2 become desphosphorylated following cell incubation with OA and LOA was increased to 20 min and 25 min respectively, when compared with untreated cells (12 min).

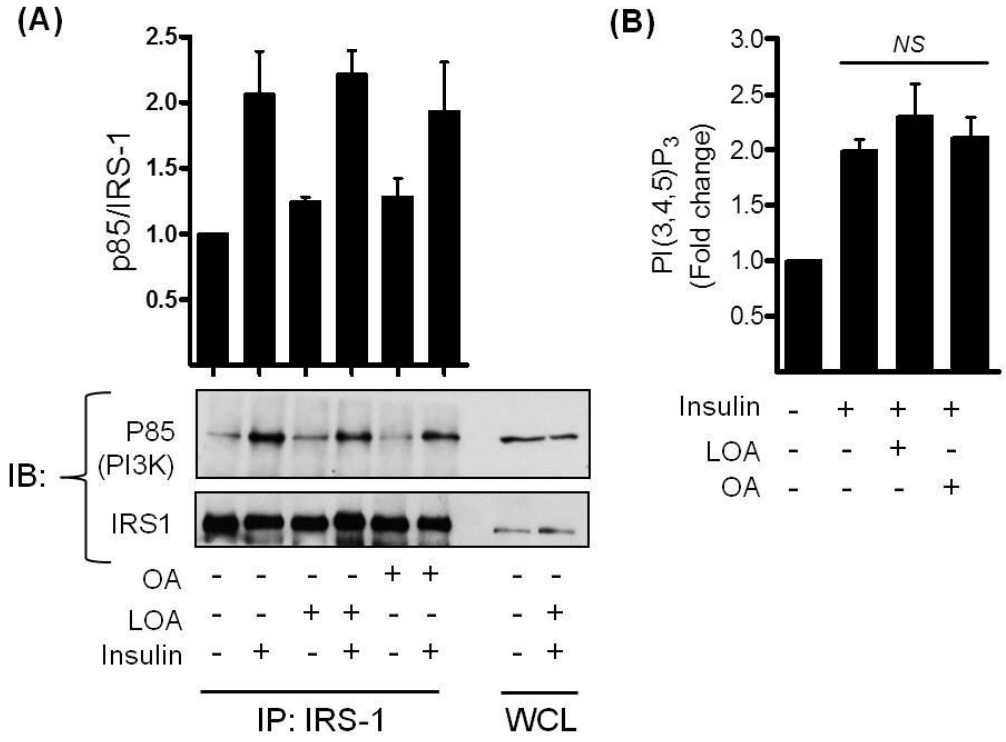


Figure 3.7 Effects of insulin, OA and LOA on p85/IRS1 association and cellular PI(3,4,5)P_3 generation in L6 myotubes.

(A, B) L6 myotubes were incubated with serum-free media containing 2% (w/v) BSA (vehicle) ± 700 µM (A) oleic acid (OA) or (B) linoleic acid (LOA) for 16 h prior to stimulation with insulin (20 nM, 10 min). (A) IRS-1 was immunoprecipitated from whole cell lysates (WCL) using an anti-IRS-1 antibody. The immunopellet was probed with an anti-p85 antibody. (B) Cells were treated as in (A) lysed and assayed for PI(3,4,5)P_3 content (Gray et al., 2003). The bar graph values (A and B) are presented as mean ± SEM of three separate experiments with *NS* indicating a no significant change between the indicated bars.

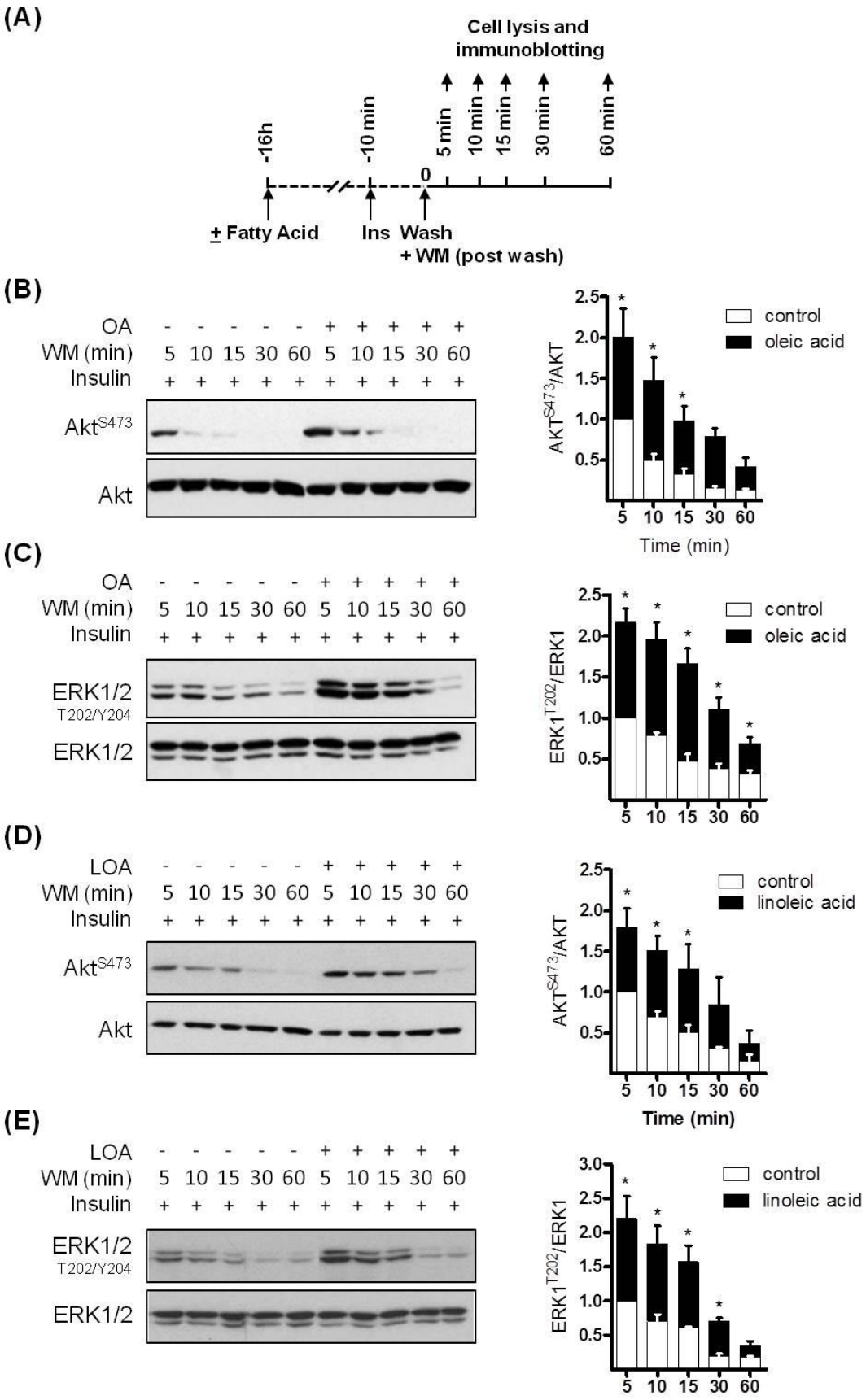

(A)
Cell lysis and immunoblotting
-16h
-10 min
0
5 min
10 min
15 min
30 min
60 min
± Fatty Acid
Ins
Wash
+ WM (post wash)
(B)
OA
WM (min)
Insulin
Akt^S473
Akt
AKT^S473/AKT
control
oleic acid
Time (min)
(C)
ERK1/2 T202/Y204
ERK1/2
ERK1^T202/ERK1
(D)
LOA
linoleic acid
(E)

Figure 3.8 OA and LOA enhance and prolong insulin-dependent phosphorylation of Akt and ERK in L6 myotubes.

The experimental approach used to test the effect of OA and LOA provision on Akt and ERK1/2 phosphorylation is depicted in **(A)**. L6 myotubes were incubated with serum-free media containing 2% (w/v) BSA (vehicle) ± 700 μM **(B, C)** oleic acid (OA) or **(D, E)** linoleic acid (LOA) for 16 h prior to stimulation with insulin (20 nM, 10 min). Cells were washed to remove insulin and subsequently incubated with 100 nM wortmannin (WM) and lysed at times indicated. Immunoblots and relevant quantification of phospho-Akt and phospho-ERK1/2 in L6 myotubes incubated with OA (B, C) or LOA (D, E). Data in the bar graphs is presented as mean ± SEM (n=3) with asterisks indicating a significant change ($P<0.05$) between the respective filled (fatty acid treatment) and unfilled (control) bar at each time point.

3.2.7 Pharmacological inhibition of protein phosphatase-2A emulates OA and LOA insulin-sensitising effect

The prolonged phosphorylation of Akt and ERK1/2 observed in presence of OA and LOA suggested that these fatty acids might affect a phosphatase targeting these kinases. Both Akt and ERK1/2 are physiological targets for PP2A (Andjelkovic et al., 1996a; Junttila et al., 2008). Therefore I hypothesised that inhibiting PP2A using okadaic acid should emulate the effect observed following exposure to OA or LOA. Using a similar strategy to that shown in Fig 3.8A, L6 myotubes were pretreated with okadaic acid (or vehicle control) for 15 min and with insulin in the penultimate 10 min period prior to washing myotubes free from okadaic acid or insulin and reincubation in media containing wortmannin for up to 60 min post-wash. Cells were lysed at time points specified in Fig 3.9A for analysis of Akt^{S473} and $ERK1/2^{T202/Y204}$ phosphorylation and reveals that pharmacological inhibition of PP2A induces heightened and sustained phosphorylation of both kinases (Fig 3.9B and C) in a manner comparable to that seen following fatty acid treatment (Fig 3.8B-E). To test whether OA and LOA further enhance the sensitising effect promoted by okadaic acid, L6 myotubes were treated with OA and LOA (700 μM) for 16 h and with increasing concentrations of okadaic acid during the penultimate 15 min of this period of incubation, prior to insulin stimulation (20 nM, 10 min). Fig 3.9D shows that PP2A inhibition by okadaic acid promotes an increase in Akt phosphorylation in a dose dependent manner. Chronic OA and LOA treatment resulted in a comparable Akt activation and no additional gain in phosphorylation was observed when myotubes were incubated with both fatty acids and okadaic acid, suggesting that they might act through the same mechanism.

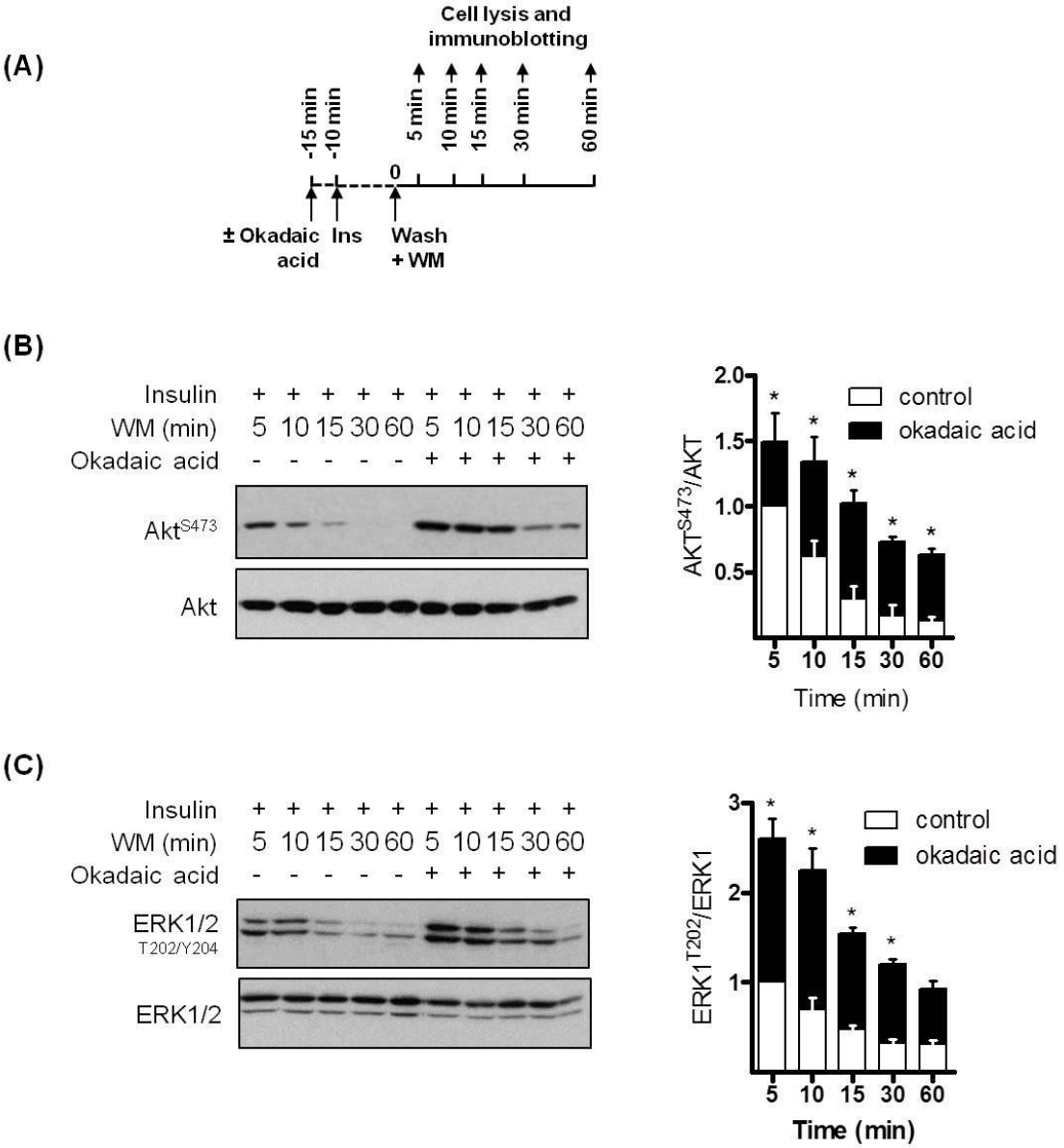

(A)
Cell lysis and immunoblotting
-15 min
-10 min
5 min
10 min
15 min
30 min
60 min
0
± Okadaic acid
Ins
Wash + WM
(B)
Insulin + + + + + + + + + +
WM (min) 5 10 15 30 60 5 10 15 30 60
Okadaic acid - - - - - + + + + +
Akt^S473
Akt
AKT^S473/AKT
control
okadaic acid
Time (min)
(C)
Insulin + + + + + + + + + +
WM (min) 5 10 15 30 60 5 10 15 30 60
Okadaic acid - - - - - + + + + +
ERK1/2 T202/Y204
ERK1/2
ERK1^T202/ERK1
control
okadaic acid
Time (min)
(D)
Akt^S473
Akt
Insulin - + + + - + + + - + + +
OA - - - - - - - - + + + +
LOA - - - - + + + + - - - -
Okadaic acid (nM) 0 0 50 100 0 0 50 100 0 0 50 100

Figure 3.9 Okadaic acid emulates the insulin-sensitising effect of OA and LOA upon Akt and ERK phosphorylation in L6 myotubes.

(A, B, C) L6 myotubes were incubated with serum-free media containing okadaic acid (100 nM) or vehicle (dH_2O) for 15 min and with insulin (20 nM) during the penultimate 10 min of this incubation. Cells were washed and then maintained in serum-free media containing wortmannin (WM, 100 nM) and lysed at times indicated. Cell lysates were subsequently immunoblotted using antibodies against the proteins indicated. Data in the bar graphs is presented as mean ± SEM (n = 3) with asterisks indicating a significant change ($P<0.05$) between the respective filled (okadaic acid treatment) and unfilled (control) bar at each time point. **(D)** L6 myotubes were incubated with serum-free media containing 2% (w/v) BSA (vehicle) ± 700 µM oleic acid (OA) or linoleic acid (LOA) for 16 h. Cells were treated with okadaic acid or vehicle (dH2O) at the concentrations indicated for 15 min and with insulin (20 nM) during the penultimate 10 min of this incubation. Cell lysates were subsequently immunoblotted using antibodies against the proteins indicated. Blots are representative of two different experiments.

3.2.8 OA and LOA increase PP2Ac inhibitory phosphorylation and demethylation

The above findings support the idea that OA and LOA may enhance insulin signalling *via* repression of PP2A. To assess this possibility I investigated the effect of OA, LOA and the SFA palmitate (PA), on PP2A. Akt-directed insulin signalling is suppressed by PA and this effect is partly reliant upon the known stimulatory effect that PA exerts upon PP2A (Blouin et al., 2010). Fig 3.10A shows that, unlike OA or LOA, sustained exposure of L6 myotubes to PA reduced insulin-stimulated Akt^{S473} phosphorylation (compare lane 2 with 4 and also lanes 6 and 8 with lane 4). This reduction appears to be antagonised when PA-treated myotubes were co-incubated with OA or LOA (compare Lane 4 with lanes 10 and 12). However, the overall gain in phosphorylation remains significantly lower when compared to myotubes exposed to OA or LOA alone (compare lanes 6 and 8 with 10 and 12, respectively).

To assess whether PP2A may be regulated in a manner that accounts for changes in Akt phosphorylation seen in response to insulin, PA, OA and LOA (Fig 3.10A) I assessed Y307 phosphorylation and carboxy-methylation of the catalytic subunit of PP2A (PP2Ac)– two different covalent modifications known to impact on PP2A holenzyme assembly and PP2A activity (Janssens and Goris, 2001). Fig 3.10B shows that compared to untreated cells, insulin was able to acutely induce $PP2Ac^{Y307}$ phosphorylation by 35%, but that this was negated in myotubes preincubated with PA. In contrast, cellular pre-treatment with OA or LOA not only induced a significant enhancement in $PP2Ac^{Y307}$ phosphorylation in response to insulin, but blocked the reduction caused by PA.

The ability of OA and LOA to enhance PP2Ac^{Y307} phosphorylation in response to insulin is not an acute event, but requires at least 9 h of cell treatment with either fatty acid to manifest (Fig 3.10C) and thus is consistent with the time course over which there is a detectable increase in insulin sensitivity as judged on the basis of Akt and ERK1/2 phosphorylation (Fig 3.2C and D). It is also noteworthy, that this fatty acid-induced increase in PP2Ac^{Y307} phosphorylation is not restricted to cultured rat muscle cells as I also observe this modulation in primary human muscle cells (Fig 3.11). Src has been implicated as the tyrosine kinase that phosphorylates PP2Ac^{Y307} and consistent with this insulin, LOA and OA all promoted Src activation based on phosphorylation of its Y416 site (Fig 3.10B). It is noteworthy that Src^{Y416} phosphorylation can be induced independently by insulin and both OA and LOA, although the latter appear slightly more potent. There appears to be no additional gain in phosphorylation of this site if myotubes are co-treated with insulin and OA or LOA (Fig 3.10D). Analysis of immunoprecipitated PP2Ac with an antibody detecting the demethylated (inactive) protein revealed that insulin enhances PP2Ac demethylation, but that this was abrogated in PA-treated myotubes (Fig 3.12A). In contrast, both OA and LOA enhanced accumulation/detection of demethylated PP2Ac that occurs with insulin and, when coincubated with PA, repress the anti-demethylation effect of the SFA. PME-1 is the enzyme responsible for PP2Ac L309 demethylation (Lee et al., 1996). Therefore, I wanted to assess whether LOA and OA promote demethylation of the phophatase by increasing expression of PME-1. However, Fig 3.12B shows that neither PA nor LOA and OA affect the mRNA level of the PME-1.

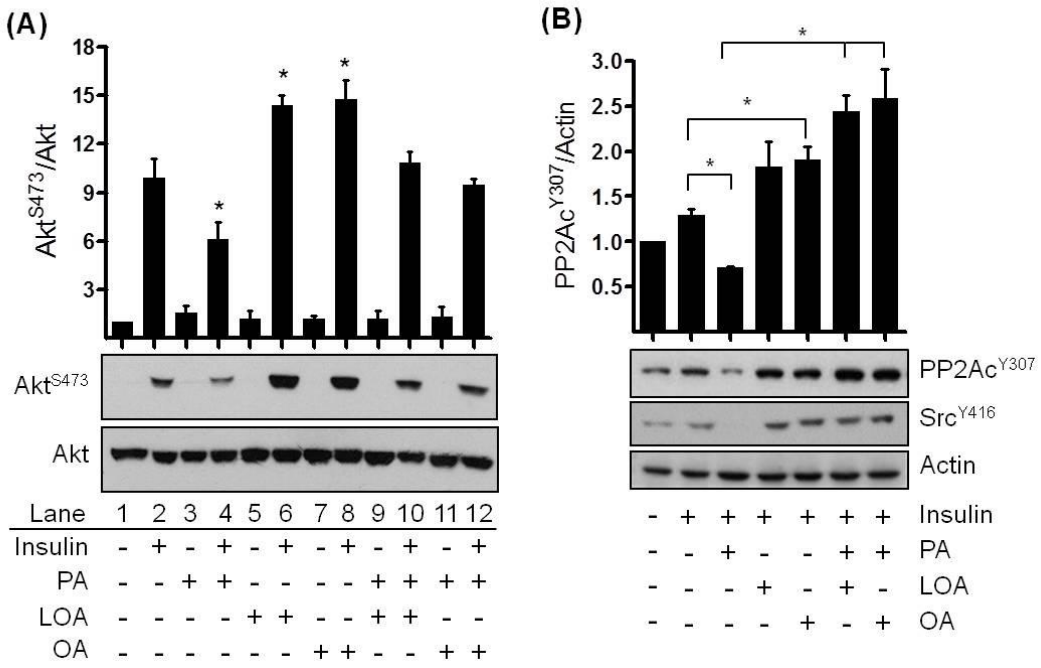

(A)
Akt^S473/Akt
18
15
12
9
6
3
Akt^S473
Akt
Lane 1 2 3 4 5 6 7 8 9 10 11 12
Insulin - + - + - + - + - + - +
PA - - + + - - - - + + + +
LOA - - - - + + - - + + - -
OA - - - - - - + + - - + +
(B)
PP2Ac^Y307/Actin
3.0
2.5
2.0
1.5
1.0
0.5
PP2Ac^Y307
Src^Y416
Actin
- + + + + + + Insulin
- - + - - + + PA
- - - + - + - LOA
- - - - + - + OA
(C)
PP2Ac^Y307
Akt
Insulin - + + + +
LOA (h) - - 3 9 16
PP2Ac^Y307
Akt
Insulin - + + + +
OA (h) - - 3 9 16
(D)
Src^Y416
GAPDH
Insulin - + - + - +
LOA - - + + - -
OA - - - - + +

Figure 3.10 Effects of insulin, PA, OA and LOA on Akt, Src and PP2Ac phosphorylation in L6 myotubes.

L6 myotubes were incubated with serum-free media containing 2% (w/v) BSA (vehicle) ± 700 µM palmitate (PA), oleic acid (OA) or linoleic (LOA) for 16 h or for times indicated. In some experiments, cells were treated with fatty acids either alone or in combination as indicated or stimulated with insulin (20 nM, 10 min) as indicated (**A-D**). Cell lysates were immunoblotted using antibodies against the proteins/ phospho-proteins indicated. Bar graphs values (A, n=3; B, n=4) are Mean ± SEM. The asterisks in (A) signify a significant change ($P<0.05$) compared to the insulin treated value (Lane 2) or between the indicated bars (B). Blots in panels C and D are representative of three different experiments.

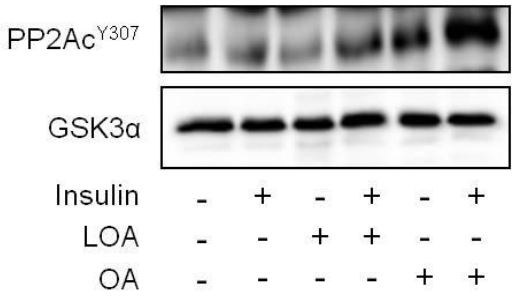


Figure 3.11 OA and LOA enhance PP2Ac phosphorylation in human myotubes.

Human myotubes were incubated with 200 µM oleic acid (OA) or linoleic acid (LOA) for 24 h prior to stimulation with insulin (20 nM, 10 min). Cell lysates were subsequently immunoblotted using antibodies against the proteins indicated. The immunoblots shown are representative of two separate experiments.

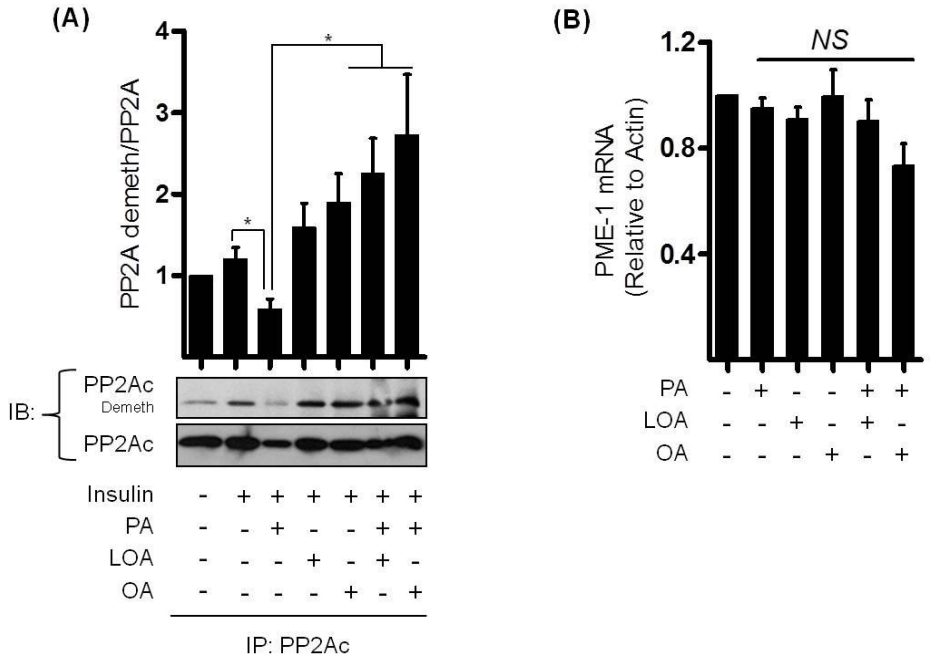


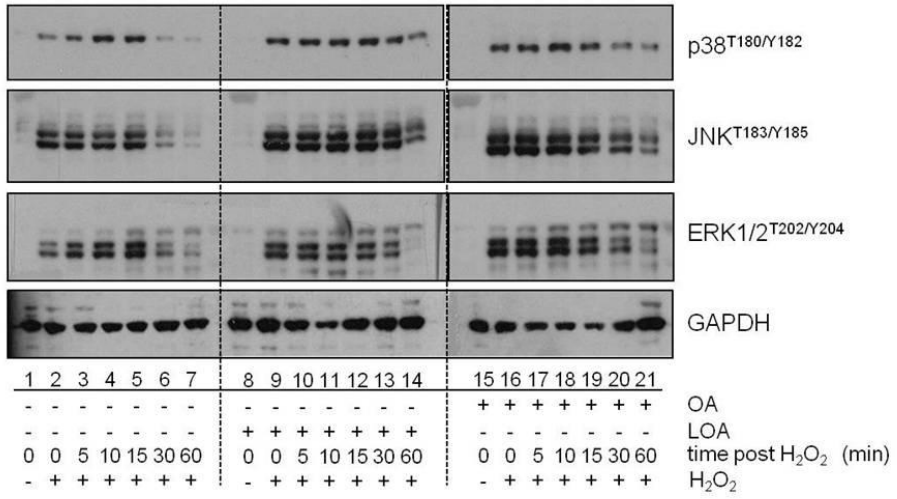
Figure 3.12 Effects of insulin, PA, OA and LOA on Akt, Src and PP2Ac demethylation in L6 myotubes.

(A, B) L6 myotubes were incubated with serum-free media containing 2% (w/v) BSA (vehicle) ± 700 µM palmitate (PA), oleic acid (OA) or linoleic (LOA) for 16 h. In some experiments, cells were treated with fatty acids either alone or in combination as indicated or stimulated with insulin (20 nM, 10 min) as indicated**.** (A) PP2Ac was immunoprecipitated from cell lysates and the immunopellet probed with an antibody recognising demethylated PP2Ac. (B) The expression of PME 1 was tested by conventional RT-PCR analysis of RNA isolated from L6 myotubes**.** Bar graphs values (A, n=7; B, n=3) are Mean ± SEM. The asterisks signify a significant change ($P<0.05$) and *NS* a no significant change between the indicated bars.

3.2.9 OA and LOA improve ERK1/2 activation by inhibiting not only PP2A but also dual specificity phosphatases

Since OA and LOA increased both ERK1^{T202} and ERK2^{Y204}, I hypothesised that their beneficial effect on signalling could be due not only to inhibition of PP2A but also that of tyrosine phosphatases. MAPKs are known to be mainly regulated by a class of dual specificity phosphatases (DUSPs), which dephosphorylate both threonine and tyrosine residue (Caunt and Keyse, 2013; Nunes-Xavier et al., 2011). To explore the possible involvement of DUSPs, L6 myotubes were incubated for 16 h with or without OA and LOA prior to stimulation of MAPK signalling with H_2O_2 (1 mM) for 15 min. After this short period of H_2O_2 stimulation, cells were washed and incubated with fresh media for periods up to 60 min, and then lysed at regular intervals to assess p38, JNK1/2 and ERK1/2 phosphorylation. As shown in Fig 3.13A, cells treated with LOA and OA exhibit greater phosphorylation/activation of MAPKs compared to myotubes not treated with fatty acid (*e.g.* compare lane 7 with lanes 14 and 21 for p38 MAPK). The result suggests that unsaturated fatty acids could reduce MAPKs dephosphorylation by inhibiting DUSPs, although additional experiments are required to confirm this hypothesis. The effect of LOA and OA on cellular tyrosine phosphorylation was also assessed. L6 myotubes were incubated with fatty acids for 16 h and insulin for 10 min and then washed as described above. As shown in Fig 3.13B, unsaturated fatty acids do not affect tyrosine phosphorylation state of the whole cell lysates indicating that fatty acids were unlikely to exert a generalised suppressive effect on tyrosine phosphatases and the effect appeared restricted to PP2A and possibly DUSPs.

(A)


p38T180/Y182
JNKT183/Y185
ERK1/2T202/Y204
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
OA
LOA
time post H2O2 (min)
H2O2
0 0 5 10 15 30 60

(B)

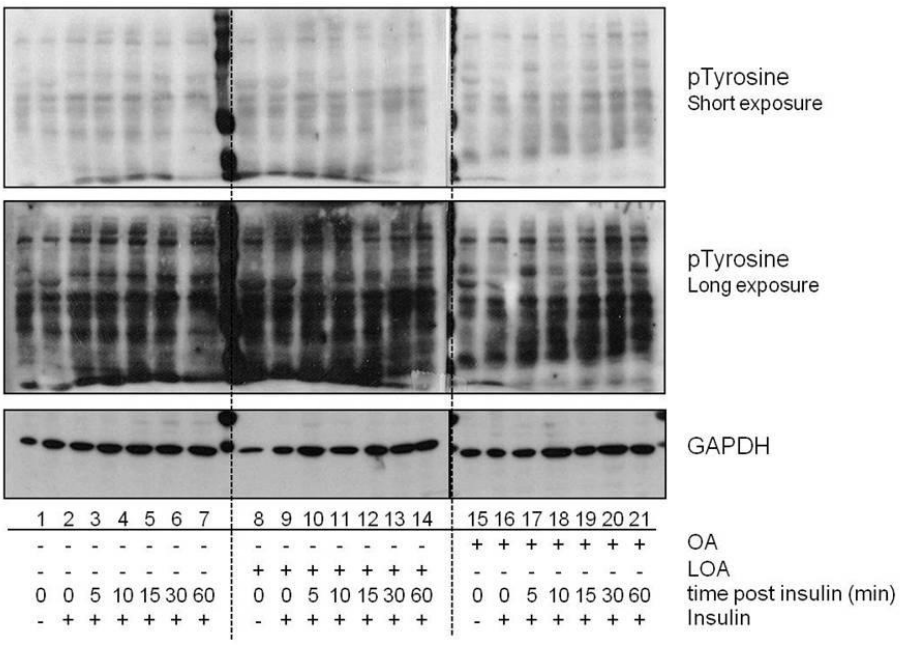

pTyrosine
Short exposure
pTyrosine
Long exposure
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
OA
LOA
time post insulin (min)
Insulin
0 0 5 10 15 30 60

Figure 3.13 OA and LOA inhibit DUSPs but not tyrosine phosphatases in L6 myotubes.

(A, B) L6 myotubes were incubated with serum-free media containing 2% (w/v) BSA (vehicle) ± 700 μM linoleic (LOA) or oleic acid (OA) for 16 h prior to stimulation with (A) H_2O_2 (1 mM) for 15 min or (B) insulin (20 nM) for 10 min. H_2O_2 and insulin were then washed off the cells and cells incubated with serum-free media for different post-wash periods indicated before being lysed. Cells lysates were subsequently immunoblotted using antibodies against proteins indicated. Blots are representative of three different experiments.

3.2.10 β-oxidation of unsaturated fatty acids is not essential to promote their beneficial effect

In order to understand whether mitochondrial transport and β-oxidation are required for the insulin sensitising effect of unsaturated fatty acids, I assessed the effect of inhibiting these processes by treatment with etomoxir (ethyl-2-[6-(4-chlorophenoxy) hexyl]-oxirane-2-carboxylate). Once taken up by cells fatty acids are activated by esterification with acyl-CoA to form fatty acyl-CoA. In order to be transported into the mitochondria to undergo β-oxidation, long-chain fatty acyl-CoA must be conjugated to carnitine by CPT-1, a protein associated with the outer mitochondrial membrane. (Longo et al., 2006). Etomoxir is a competitive inhibitor of CPT-1, the rate limiting enzyme for β-oxidation, and therefore represents an efficient strategy for blocking fatty acid oxidation (Agius et al., 1991; Declercq et al., 1987). L6 myotubes were incubated, in absence or presence of etomoxir (100 µM), for 16 h with OA and LOA alone or combined with PA (700 µM), prior to stimulation with insulin (20 nM) for 10 min (Fig 3.14.A). Interestingly, cell treatment with etomoxir did not affect the ability of unsaturated fatty acids to enhance insulin sensitivity of skeletal muscle cells suggesting that their mitochondrial oxidation is not essential for their insulin-sensitising effect. In contrast, suppressing mitochondrial fatty acid oxidation exacerbated the insulin-desensitising effect of PA as demonstrated by further loss in Akt phosphorylation (compare lanes 3 and 10, Fig 3.14A). Studies from the last two decades have shown that the development of insulin resistance is associated with reduced fat oxidative capacity (Kelley and Simoneau, 1994; Phielix and Mensink, 2008). Reduced PA oxidation would promote greater partitioning of the fatty acids towards synthesis of intermediates, such as DAG,

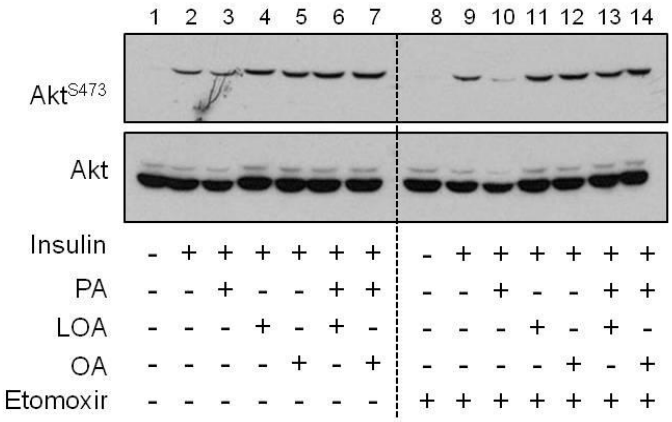


Figure 3.14 OA and LOA β-oxidation is not necessary to promote insulin sensitising effect in in L6 myotubes.

(A) L6 myotubes were incubated with serum-free media containing 2% (w/v) BSA (vehicle) ± 700 µM linoleic (LOA) or oleic acid (OA) for 16 h, in presence or absence of etomoxir (100 µM). In some cases, cells were treated with palmitate (PA) (700 µM) for 16 h alone or in combination with LOA or OA. Cells were then stimulated with insulin (20 nM) for 10 min as shown, lysed and immunoblotted using antibodies against proteins indicated. Blots are representative of three different experiments.

which are responsible for lipotoxicity and consequent development of insulin resistance. Importantly, OA and LOA can still attenuate PA inhibitory effect on insulin signalling in the presence of etomoxir, suggesting that MUFAs and PUFAs retain their protective effect and limit lipotoxicity despite their reduced oxidation.

3.2.11 The insulin sensitising effect of OA and LOA does not involve caveolin1/3 proteins

Several studies have shown that dietary fatty acid can alter membrane lipid composition, which may be of particular significance for lipid rafts and caveolae (Li et al., 2007; Ma et al., 2004), as these have been suggested to play an important role in the compartmentalisation, modulation and integration of many distinct signalling cascades. Experiments performed in our laboratory have demonstrated, for example, that silencing the expression of caveolin 1 and 3 in L6 skeletal muscle cells is associated with reduced activation/phosphorylation of Akt in response to insulin. Given the ability of caveolins to influence insulin signalling, I investigated whether the beneficial effect of OA and LOA on insulin action may depend on caveolar integrity and function as assessed by lentiviral mediated gene knockdown of caveolin 1 and 3 in L6 cells. Control cells (infected with lentiviral particles harbouring a scrambled shRNA insert) and caveolin 1/3 knockdown (kd) L6 cells were treated for 16 h with LOA and OA prior to stimulation with insulin (20 nM) for 10 min. As shown in Fig 3.15A, Cav1/3 kd cells present a decreased Akt and ERK1/2 phosphorylation (compare lane 2 with 8), confirming that reduced caveolins expression results in induction of insulin resistance (Cohen et al., 2003; Oh et al., 2008). However, OA and LOA can still improve Akt and ERK1/2 activation, suggesting that their insulin

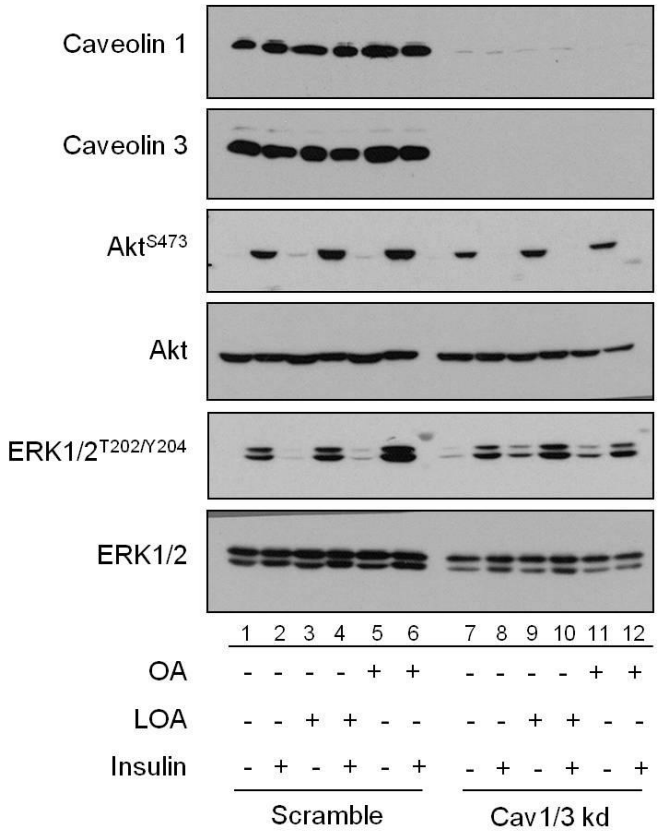


Figure 3.15 The insulin sensitising effect of OA and LOA is not dependent upon caveolin 1 and 3 expression in L6 myotubes.

(A, B) Fully differentiated stable cav1/3 knockdown L6 cells along with corresponding scramble control cells were incubated with serum-free media containing 2% (w/v) BSA (vehicle) ± 700 µM oleic acid (OA) or linoleic acid (LOA) for 16 h, prior to insulin stimulation (20 nM) for 10 min. Cell lysates were then analysed by immunoblotting with antibodies against proteins indicated. Blots are representative of three separate experiments.

sensitising effect is unlikely to be dependent upon caveolin 1 and 3.

3.2.12 AMPK is not involved in the modulation of OA and LOA-induced insulin sensitivity

A recent study has demonstrated that MUFAs improve insulin sensitivity and prevent PA-induced insulin resistance through an AMPK-dependent mechanism (Salvado et al., 2013). AMPK is a potent regulator of skeletal muscle metabolism, by regulating several intracellular systems including glucose uptake, fatty acid oxidation, biogenesis of GLUT4 and mitochondria (Hardie et al.; Jorgensen et al., 2004). The heterotrimeric AMPK holoenzyme consists of one catalytic subunit (α) and two functionally and structurally different regulatory subunits (β, γ). The catalytic subunit is present as 2 isoforms, a universally expressed α_1 isoform and an α_2 isoform (O'Neill, 2013). Since AMPK plays an important role in promoting glucose uptake and preventing insulin resistance (Fujii et al., 2008; Jorgensen et al., 2004), I investigated whether this protein kinase may mediate the beneficial effects of OA and LOA. Fig 3.16A shows that in skeletal muscle cells, as previously seen in other tissues (Horman et al., 2006; Kovacic et al., 2003; Minokoshi et al., 2004), insulin stimulation inhibits AMPK activation- the down-regulation appears more evident based on phosphorylation analysis of its downstream target ACC. In sharp contrast, chronic treatment with fatty acids resulted in increased phosphorylation of $AMPK^{T172}$ and ACC^{S59}. To further investigate the role of AMPK in the fatty acid-induced insulin sensitising effect, L6 myotubes in which AMPK α1 had stably been silenced were incubated for 16 h with the OA and LOA prior to stimulation with insulin (20 nM) for 10 min. Silencing the AMPK α1 catalytic subunit led to a decreased insulin-stimulated Akt phosphorylation compared to control cells

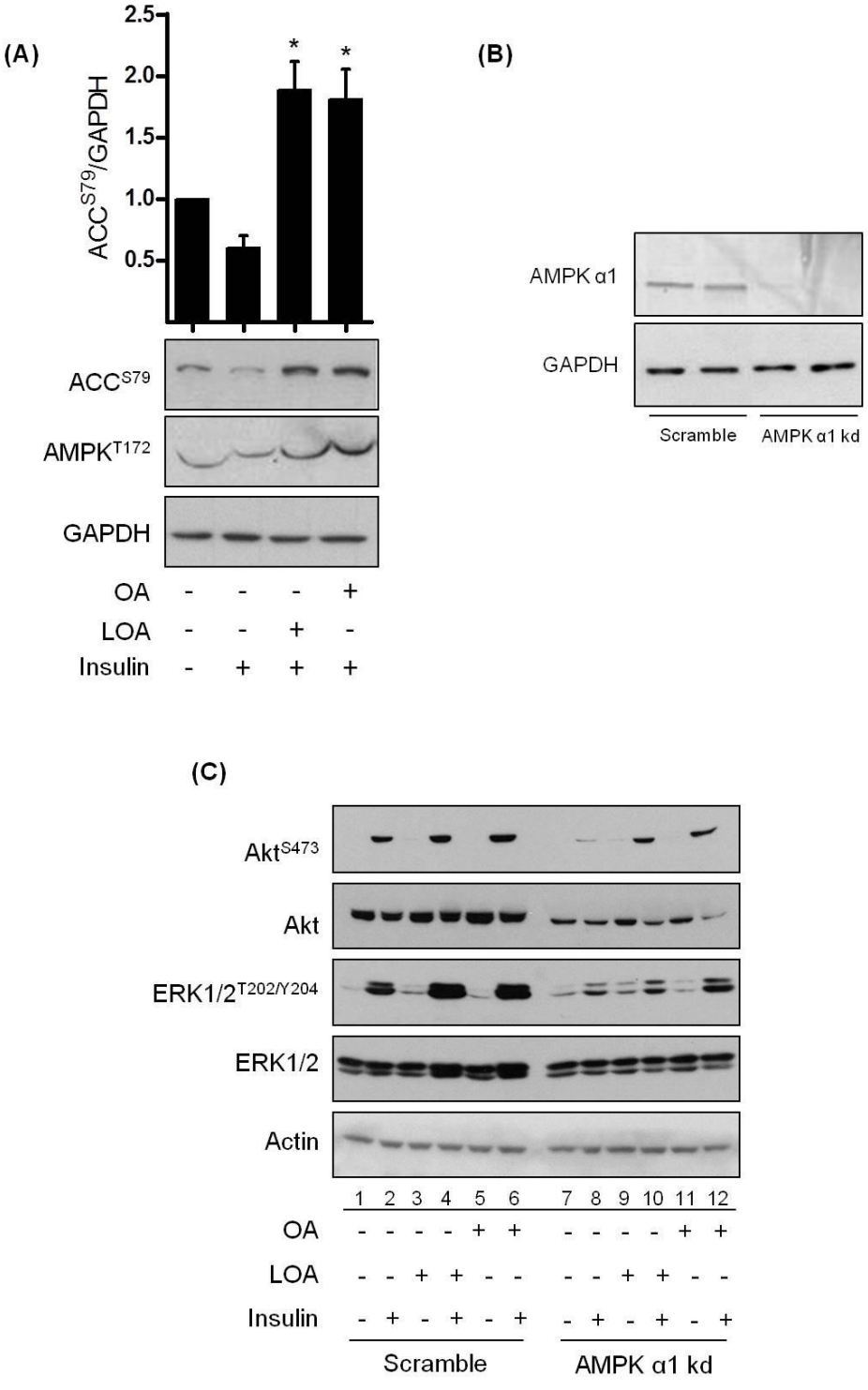

(A)
2.5
2.0
1.5
1.0
0.5
ACC^{S79}/GAPDH
*
*
ACC^{S79}
AMPK^{T172}
GAPDH
OA - - - +
LOA - - + -
Insulin - + + +
(B)
AMPK α1
GAPDH
Scramble
AMPK α1 kd
(C)
Akt^{S473}
Akt
ERK1/2$^{T202/Y204}$
ERK1/2
Actin
1 2 3 4 5 6 7 8 9 10 11 12
OA - - - - + + - - - - + +
LOA - - + + - - - - + + - -
Insulin - + - + - + - + - + - +
Scramble
AMPK α1 kd

Figure 3.16 AMPK α1 is not involved in the modulation of insulin sensitising effect by OA and LOA in L6 myotubes.

(A) L6 myotubes were incubated with serum-free media containing 2% (w/v) BSA (vehicle) ± 700 μM linoleic (LOA) or oleic acid (OA) for 16 h, prior to insulin stimulation (20 nM) for 10 min. Cell lysates were then analysed by immunoblotting with antibodies against proteins indicated. Data in the bar graphs is presented as mean ± SEM (n = 3) with asterisks indicating a significant change ($P<0.05$) relative to the insulin treatment alone. **(B)** Lentiviral mediated gene knockdown (kd) of AMPK α1 in L6 cells. **(C)** Stable AMPK α1 knockdown in fully differentiated L6 cells along with corresponding control cells (infected with scramble shRNA construct) were incubated with serum-free media containing 2% (w/v) BSA (vehicle) ± 700 μM linoleic (LOA) or oleic acid (OA) for 16 h, prior to insulin stimulation (20 nM) for 10 min. Cell lysates were then analysed by immunoblotting with antibodies against proteins indicated. Blots are representative of three separate experiments.

infected with the scrambled shRNA construct (Fig 3.16C, compare lane 2 with 8), although it is noterworthy that total Akt expression was also reduced in these cells. In any event the unsaturated fatty acids were still able to sensitise L6 myotubes to insulin (Fig 3.16C), suggesting that AMPK was not an essential requirement for mediating their insulin sensitising effect. A previous study has shown that high-fat diet α_2 Tg mice presented reduced IRS1 and Akt protein level in skeletal muscle compared to the control, which contributed to a reduced insulin-stimulated glucose uptake (Fujii et al., 2008); anyway the molecular mechanism by which this effect could occur is still unclear.

3.3 Discussion

Numerous studies in the literature have shown that increased availability of SFAs is linked to a decreased insulin sensitivity in insulin-target tissues, with important knock-on consequences for processes such as gene expression and cell metabolism that are normally regulated by the hormone (Lipina and Hundal, 2010). In contrast, considerable evidence supports the view that development of insulin sensitivity is positively correlated with supply of MUFAs and PUFAs and that, in many instances, their provision not only promotes beneficial metabolic changes but negates some of the harmful effects associated with SFA oversupply (Coll et al., 2008; Dimopoulos et al., 2006; Macrae et al., 2013). Since skeletal muscle is responsible for 75-80% of insulin-stimulated glucose uptake in a healthy individual, it follows that this tissue is the more affected by elevation in circulating free fatty acids and their effect upon insulin signalling (Manco et al., 2004). Indeed, studies carried out in both animals and humans have shown that the fatty acid composition of stored and structural lipids in skeletal muscle cells reflects the intake of dietary fat and is related to peripheral insulin sensitivity (Andersson et al., 2002). Therefore, although it is still unclear how unsaturated fatty acids act on insulin signalling, the sensitising effect performed by them could be due mainly to their effect on skeletal muscle. The major aim of this study was to analyse the effects of different MUFAs and PUFAs on L6 rat skeletal muscle cells and gain further insight of how unsaturated fatty acids can prevent some of the detrimental effects promoted by SFAs.

The results obtained showed that incubation of muscle cells with MUFA oleic (18:1, n-9), and with PUFAs linoleic (18:2, n-6) and α-linolenic (18:3, n-3)

induces an increase in the insulin-stimulated phosphorylation of Akt and MAPK ERK1/2. In contrast, treatment with γ-linolenic acid (a PUFA, 18:3, n-6) impairs Akt activation (Fig 3.1). Since the fatty acids used are all of 18 carbon chain-length, it follows that the position of double bonds along the chain could have a bearing upon their insulin-sensitising effect thus explaining the opposite effect elicited by γ-linoleic acid. Among all of the unsaturated fatty acids used for treatment, linoleic acid (LOA) and oleic acid (OA) have been shown to promote the greatest positive effect upon insulin signalling. Interestingly, the improved insulin sensitivity elicited by these unsaturated fatty acids was most prominent when cells were stimulated with a sub-maximal concentration of insulin. The sensitisation was not apparent upon challenging cells with a 100 nM insulin stimulation, which may explain why other studies (using maximally effective insulin concentrations) did not report beneficial gains in Akt activation by insulin in the presence of unsaturated fatty acids (Dimopoulos et al., 2006; Yuzefovych et al., 2010). Indeed, previous work from the Hundal lab has shown that provision of MUFAs (*e.g.* palmitoleate and oleic acid) can induce insulin-like effects upon distal cellular responses, such as glucose uptake by inducing the plasma membrane recruitment of glucose transporters (Dimopoulos et al., 2006); however, no additional enhancement in glucose uptake is observed when muscle cells are simultaneously challenged with the MUFA and a maximally effective concentration of insulin (Dimopoulos et al., 2006).

Given that the sensitising effect of unsaturated fatty acids on the Akt pathway is a chronic response that develops over a 16 h period, I hypothesised that LOA and OA may act by increasing expression of upstream signalling proteins of insulin pathway. However, protein expression of the insulin receptor (IR), insulin

receptor-substrate 1 (IRS1) and PI3K p85 were not influenced by chronic treatment with LOA and OA (Fig 3.4). Moreover, the interaction between IRS1 and p85-PI3K and PI(3,4,5)P_3 lipid production, events necessary for Akt activation, were unaffected by provision of unsaturated fatty acids (Fig 3.6). The finding that unsaturated fatty acids do not affect the tyrosine phosphorylation state within whole cell lysates (Fig 3.13B) is consistent with this finding, thus excluding the possibility that enhancement of insulin signalling may has been a direct consequence of enhanced insulin receptor tyrosine phosphorylation.

Intriguingly, I did not observe any increase in mTOR activation, which is stimulated not only by nutrient provision but also by insulin (Bolster et al., 2004). Indeed, activation of P70S6K, a direct target of mTOR, by insulin was not enhanced by cell incubation with OA and LOA, despite increased phosphorylation of Akt, which has been suggested to stimulate mTOR signalling. This effect might be due to Akt-mediated inhibition of GSK3. Shin *et al.* have reported that GSK3 positively regulates P70S6K *via* S371 phosphorylation (Shin et al., 2011). The S371 residue is located within a turn motif and is required for mTOR-mediated T389 phosphorylation and consequent P70S6K activity (Dann et al., 2007; Fingar and Blenis, 2004). Therefore, by promoting GSK3 inhibition through Akt, LOA and OA might negate any increase in P70S6K activation. In addition, my data indicate that OA and LOA provision does not induce modulation of mTOR/P70S6K axis in response to amino acid provision, suggesting that unsaturated fatty acids do not influence this pathway in response to acute nutrient stimulation.

Since the beneficial effect exerted by LOA and OA cannot be attributed to an increased activation of upstream components of the insulin signalling cascade,

it follows that the inhibition of a phosphatase that targets AKT and ERK1/2 may be a possible mechanism. Among the phosphatases involved in regulation of insulin signalling, protein phosphatase-2A, PP2A, can dephosphorylate both Akt and ERK1/2 (Cazzolli et al., 2001; Ruvolo, 2003; Ugi et al., 2004; Van Kanegan et al., 2005). PP2A is a serine/threonine phosphatase that accounts for ~1% of total cellular proteins and plays an important role in many cellular processes, including proliferation, cell death and regulation of cell cycle (Janssens and Goris, 2001). Moreover, evidence in the literature demonstrates that PA and its derivative ceramide can positively regulate the expression and activity of this phosphatase resulting in a suppressive effect upon insulin signalling (Cazzolli et al., 2001; Ruvolo, 2003). Therefore, the sensitising effect exerted by LOA and OA may involve suppression of PP2A. This proposition is based on the following lines of evidence. First, while sustained exposure of muscle cells to OA and LOA by themselves have no effect on Akt and ERK1/2 both fatty acids significantly enhance phosphorylation of these kinases and their downstream targets in response to an acute sub-maximal insulin challenge in a PI3K-independent manner. Second, inhibition of PP2A mimics the enhanced and sustained phosphorylation pattern seen for Akt and ERK1/2 following cell treatment with OA or LOA. Third, both unsaturated fatty acids promote PP2A tyrosine phosphorylation and demethylation. The latter is relevant as carboxymethylation of L309 on PP2Ac is required for assembly of the PP2A holoenzyme and for catalytic activation, whereas phosphorylation of Y307 inhibits PP2A catalytic activit (Begum and Ragolia, 1996; Chen et al., 1992).

Several groups have reported hyperactivation of PP2A in response to sustained oversupply of glucose and SFAs (*i.e*. glucolipotoxicity), which may be important

in the pathogenesis of insulin resistance (Galbo et al., 2011; Kowluru and Matti, 2012; Kowluru and Metz, 1997; Wu et al., 2007). Such hyperactivation may not only involve an increase in PP2Ac expression but that also of the many regulatory PP2A subunits (Eichhorn et al., 2009), a proposition based on the finding that Ptpa, B55α and B56β (which encode PP2A regulatory subunits) are all elevated in muscle and liver of insulin resistant (ZDF) rats (Galbo et al., 2011). Whilst I cannot discount the possibility that OA and LOA may repress expression of the catalytic and certain regulatory PP2A subunits, my finding that they restrain PA-induced changes in PP2Ac phosphorylation/methylation is likely to be of significance in helping to counter PP2A hyperactivation by SFAs. (Blouin et al., 2010; Chen et al., 1992; Eichhorn et al., 2009; Kowluru and Matti, 2012).

The differential effect that unsaturated and saturated fatty acids have upon PP2A may be explained, in part, by their contrasting effects on Src, which affects PP2A activity *via* direct phosphorylation of PP2Ac at Y307. In line with previous studies (Feng et al., 2012), my findings indicate that PA reduces Src activation based on a striking reduction in Src^{Y416} phosphorylation that leads to an associated reduction in the downstream phosphorylation of PP2Ac on Y307 (Fig 3.10B). As a consequence, PP2A would be expected to be more active under these circumstances leading to the counter-modulation of signalling molecules activated by insulin such as Akt and ERK1/2. In sharp contrast, both OA and LOA induced Scr^{Y416} phosphorylation to a level comparable if not greater than that seen in response to insulin that promotes a concomitant increase in PP2Ac tyrosine phosphorylation (Fig 3.10B). It is noteworthy that the ability of OA and LOA to promote Src activation is not unprecedented and has

been previously reported in both human breast cancer cells and mouse gustatory cells in which activation of Src was linked to increases in ERK1/2 activation and calcium signalling, respectively (El-Yassimi et al., 2008; Soto-Guzman et al., 2008). Unlike PA, the modulation of Src by OA and LOA in skeletal muscle cells would suppress the action of PP2A leading, as I show, to greater and more sustained activation of some of its targets (*i.e.* Akt and ERK) by insulin. The molecular basis for this differential fatty acid effect on Src is currently unclear but may depend upon their ability to activate membrane bound fatty acid receptors, such as GPR40 and GPR120, that couple to signalling molecules influencing the activation status of Src. Indeed, long and medium-chain fatty acids have been identified as ligands for these receptors, which may be involved in ameliorating insulin resistance (Talukdar et al., 2011). In particular, it has been demonstrated that GPR120 expression in adipocytes is required for allowing the beneficial effects of omega-3 (ω-3) PUFAs to be expressed in conditions characterised by chronic inflammation and insulin resistance (Oh et al., 2010). In contrast, the role of GPR40 in mediating insulin-sensitising effects remains controversial. Some studies have demonstrated that mice deficient in GPR40 are protected from obesity-induced hyperglycemia and glucose intolerance (Steneberg et al., 2005), whereas others have shown that loss of GPR40 promotes obesity, glucose intolerance and insulin resistance (Kebede et al., 2008). Nonetheless, there is evidence in the literature that suggests GPR40 is involved in mediating the effects of unsaturated fatty acids upon glucose-stimulated insulin secretion from pancreatic β-cells (Schnell et al., 2007). Moreover, activation of such a receptor has been implicated in OA-induced activation of ERK1/2 and AP-1 DNA binding activity by a mechanism

involving Src activation in MCF-7 breast cancer cells (Yonezawa et al., 2004). Whether such a mechanism may also explain activation of Src by OA and LOA in skeletal muscle cells is currently unknown, although I do have evidence that GPR40 (but not GPR120) is expressed in our cultured L6 myotubes. If GPR40 is indeed involved in initiating changes in insulin sensitivity in response to increased availability of MUFAs and PUFAs then my findings would suggest that either PA is not a ligand for GPR40 or that both OA and LOA have far greater efficacy for the receptor than the SFA in skeletal muscle cells. Testing these latter possibilities and defining whether GPR40 is a component of the mechanism by which OA and LOA enhance skeletal muscle insulin-sensitivity represent important investigative goals of future work.

While my findings indicate that both OA and LOA promote an increase in proximal insulin signalling and are able to attenuate the effect that PA has upon Src and PP2A they do not confer complete protection against the insulin-desensitising effect that PA has, for example, upon Akt activation (Fig 3.10A). Recent work from the Hundal lab has demonstrated that PA uptake into muscle cells is not affected by coprovision of MUFAs and that under these circumstances there is no reduction in PA-derived synthesis of ceramide (Macrae et al., 2013). Consequently, sustained generation of this sphingolipid will activate atypical PKCs that are known to physically associate with and negatively regulate Akt activation (Powell et al., 2003; Powell et al., 2004). However, despite the inability to restrain ceramide synthesis from the SFA it is apparent that provision of MUFAs and PUFAs do nonetheless help mitigate against some of the deleterious effects that PA has upon insulin-action *via*, for example, its ability to activate PP2A.

It is important to stress that such mitigation is not solely dependent upon counter-regulation of PP2A. In addition to increases in the intramyocellular abundance of lipids such as ceramide and DAG, sustained over-supply of PA promotes expression of pro-inflammatory genes (*e.g.* IL-6, TNFα) (Green et al., 2011), induces ER stress (Hage Hassan et al., 2012; Salvado et al., 2013) and mitochondrial dysfunction, which, increasingly, is being seen as a major factor contributing to the development of muscle insulin resistance (Muoio and Neufer, 2012; Turner and Heilbronn, 2008). The Hundal group has very recently demonstrated that sustained increases in PA promote a reduction in the expression of key mitochondrial respiratory proteins (SDHA and COX1V) and that of PGC1α which plays a major role in regulating mitochondrial biogenesis (Lipina et al., 2013; Macrae et al., 2013). A critical consequence of these changes is a reduction in respiratory drive, which will promote incomplete oxidation of fatty acids and accumulation of acylcarnitines, which have also been implicated as a cause of insulin resistance by mechanisms that currently remain unclear (Koves et al., 2008). Importantly, however, reducing the carbon load on mitochondria by withdrawing glucose or inhibiting its metabolism or coprovision of palmitoleate is found to strongly antagonise the SFA-induced reduction in PGC1α, SDHA and COX1V expression, which results in more efficient oxidation and/or partitioning of fatty acids into neutral triglyceride (Lipina et al., 2013; Macrae et al., 2013) with beneficial gains in both insulin sensitivity and metabolic status (Lipina et al., 2013). My data showed that LOA and OA can still antagonise PA detrimental effect upon insulin signalling in presence of a CPT1-inhibitor, suggesting that they rather enhance partionioning of the fatty acid into neutral lipids (Fig 3.14B-C). However, OA and LOA may

also modulate fatty acid mitochondrial oxidation since chronic treatment of muscle cells with these unsaturated fatty acids result in an enhanced phosphorylation of AMPK and its downstream target ACC (Fig 3.16A). ACC is a modulator of fatty acid mitochondrial oxidation, since it promotes production of malonyl CoA, a potent inhibitor of CPT-1 (Ruderman et al., 1999). As such, phosphorylation of ACC by AMPK will result in its inhibition and a concomitant reduction of malonyl CoA, facilitating, in turn, mitochondrial fatty acid uptake. Another potential mechanism by which provision of unsaturated fatty acids may bring about these favorable changes in mitochondria may involve their ability to bind and activate members of the peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors, which, along with PGC1α, regulate genes whose products have been shown to influence fuel and energy metabolism (Muoio and Neufer, 2012).

As mentioned earlier, PA over-supply promotes pro-inflammatory signalling and cytokine production in insulin target tissues (Ajuwon and Spurlock, 2005; Green et al., 2011; Joshi-Barve et al., 2007). It is now clear in fact that obesity, characterised by increased levels of circulating SFAs, is associated with a low grade inflammation, which contributes to the development of insulin resistance (Attie and Scherer, 2009; Lumeng and Saltiel, 2011). Whilst, PA is able to instigate proinflammatory signalling, cell exposure to MUFAs such as palmitoleate or oleate does not induce similar cytotoxic or proinflammatory effects. Indeed, MUFAs are able to restrain the proinflammatory effect of SFAs (Coll et al., 2008; Kwon et al., 2014; Macrae et al., 2013). Previous work has suggested that activation of AMPK by MUFAs and ω-3 PUFAs may contribute to the observed anti-inflammatory effect (Green et al., 2011; Macrae et al.,

2013; Xue et al., 2012). As such, the finding that OA and LOA enhance AMPK activation would suggest that, the kinase may contribute, in part, towards beneficial effect of LOA and OA by preventing PA-induced inflammatory effect. However, it remains unclear how important AMPK is for the overall improvement in insulin-signalling especially in light of the finding that α1-AMPK silencing appeared to have no impact on the OA and LOA insulin-sensitising response in L6 myotubes.

Another potential mechanism by which MUFAs and PUFAs may exert their beneficial effects is *via* activation of cellular fatty acid sensors such as Ubxd8, which respond to graded increases in unsaturated fatty acids and support greater triglyceride synthesis (Lee et al., 2010). It is plausible that by increasing partitioning of fatty acids into neutral lipid that this produces beneficial changes in metabolic status (by reducing the carbon load on mitochondria) and cellular sensitivity to insulin.

In summary, the enhanced insulin signalling associated with supply of fatty acids such as OA and LOA is likely to reflect their ability to repress action of phosphatases such as PP2A (*via* modulation of upstream kinases like Src and that of enzymes mediating carboxymethylation of PP2Ac), but potentially are also contingent on benefits accrued, for example, by activation of PPARs that facilitate more efficient metabolic handling of fatty acids especially those that are likely to impose a heavy burden on the metabolic/oxidative machinery. My observations would support the view that diets enriched with OA and/or LOA may confer important insulin-sensitising and metabolic benefits. Indeed, a recent study assessing dietary fatty acid composition and insulin sensitivity in young adults found that replacing dietary PA with OA benefits clinically relevant

measures of metabolic wellbeing, including insulin sensitivity (Kien et al., 2013a). Although the precise molecular events that underpin this benefit remain unclear, the authors did report that unlike those on the OA diet, those on the PA-enriched diet had more of their fatty acid routed towards production of acylcarnitines and ceramide consistent with the idea that PA promotes greater metabolic dysfunction, whereas OA is protective in this regard.

Chapter 4

Fatty acid modulation of neutral amino acid transporter SNAT2 in L6 myotubes and HeLa cells

4.1 Introduction

SNAT 2 is the most widely expressed member of the “System A” SLC38 family of amino acid transporters, which mediate the cellular uptake of short chain neutral amino acids and are characterised by their ability to mediate uptake of *N*-methylated amino acid substrates, such as Me-AIB (Mackenzie and Erickson, 2004). Amino acid uptake *via* SLC38 transporters is coupled to the inward movement of sodium ion down its electrochemical gradient, which promotes an outwardly-directed concentration gradient for System A substrates that can be utilised to drive the exchange uptake of a range of essential amino acids (*e.g.* leucine) through transporters (such as System L) that function in parallel with SLC38 in the plasma membrane (Baird et al., 2009; Hundal and Taylor, 2009). This amino acid exchange arrangement is considered pivotal for sensing of amino acids upstream of mTORC1 (the mammalian target of rapamycin complex 1) and since SNAT2 itself is subject to extensive regulation by growth factors, hormones (*e.g.* IGF-1 and insulin), amino acid availability as well as osmotic stress (see reviews (Hundal and Taylor, 2009; Mackenzie and Erickson, 2004)), its activity not only influences mTOR signaling (Pinilla et al., 2011) but controls diverse amino acid-dependent processes that impact on cell, tissue and whole body function (Hundal and Taylor, 2009).

A key conserved cellular trait is the ability of SNAT2 to be upregulated in response to extracellular amino acid limitation, a process known as adaptive regulation (Hyde et al., 2003; Kilberg et al., 2005). Sustained periods of extracellular amino acid deprivation result in upregulation of SNAT2 expression/function by a mechanism partly sensitive to inhibitors of RNA and protein synthesis (Gazzola et al., 1981; Hyde et al., 2001). The transcriptional

upregulation of SNAT2 in response to amino acid withdrawal relies upon a tripartite amino acid response element in the first intron of the *SLC38A2* gene (Palii et al., 2006). Precisely how an increase in SNAT2 transcription is triggered by amino acid deficiency remains unclear, although genetic interventions and use of pharmacological inhibitors have implicated the GCN2/ATF4 pathway (Kilberg et al., 2005) and members of the MAP kinase family (ERK1/2 and JNK) although, as yet, the nutrient signalling loci that lie upstream of MAPKs remain unidentified (Franchi-Gazzola et al., 1999; Lopez-Fontanals et al., 2003). Whilst increased SNAT2 transcription contributes to the overall increase in SNAT2 abundance, previous work from the Hundal lab has shown that the SNAT2 adaptive response also includes a non-genomic component involving enhanced stabilization of the SNAT2 protein (Hyde et al., 2007). It is thought that under amino acid deficient conditions SNAT2 adopts a structurally more stable configuration, whereas SNAT2 occupancy by any one single amino acid substrate is sensed as reflecting a state of amino acid sufficiency and one that signals a reduction in SNAT2 transcription and associated destabilization/loss of SNAT2 protein (Hyde et al., 2007). Consequently, SNAT2 is thought to function as an amino acid sensor or "transceptor" with the capacity to signal to nutrient responsive pathways that impact upon gene expression and protein turnover.

Although numerous studies have explored the processes by which SNAT2 is upregulated in response to amino acid deprivation, our knowledge of the mechanisms that induce SNAT2 degradation by contrast remain poorly investigated. One potential process that may serve as an important determinant of SNAT2 turnover is the ubiquitin proteasome system (UPS) given that

Nedd4.2, a ubiquitin E3-ligase, has previously been implicated in the polyubiquitination and degradation of SNAT2 (Hatanaka et al., 2006). No information currently exists of how SNAT2 processing *via* the UPS may be stimulated but given that fatty acid-derived lipids such as ceramide promote a reduction in cell surface SNAT2 with a concomitant loss in membrane transport activity (Hyde et al., 2005), it is plausible that SNAT2 processing *via* the UPS may be subject to regulation by fatty acid availability. Indeed, evidence showing that fatty acids (*e.g.* oleic and linoleic acid) can stimulate proteolytic activity in muscle (Dahlmann et al., 1985) and promote proteasomal degradation of membrane (*e.g.* CD36 (Smith et al., 2008), tyrosinase (Ando et al., 2004)) and cytosolic proteins (*e.g.* fatty acid synthase (Wojcik et al., 2014)) provide strong support for this hypothesis.

In this chapter, I investigated the effect of linoleic acid; (LOA, a PUFA (C18:2, ω-6)), oleic acid (OA, a MUFA (C18:1, ω-9)) and palmitate (PA, a SFA (C16)) upon the SNAT2 adaptation response to osmotic stess and/or amino acid withdrawal. I demonstrate that, whilst amino acid deprivation induces a robust increase in cellular SNAT2 expression and function, this adaptive increase in SNAT2 can be significantly suppressed by pretreatment of cells with LOA, OA and PA, with the PUFA and the SFA having a more potent effect than the MUFA. This fatty acid-induced suppression in SNAT2 adaptation cannot be attributed to reduced transcription of the *SLC38A2* gene but rather is a consequence of enhanced loss of SNAT2 protein abundance at the surface. However, PA and LOA appear to act through different molecular mechanisms. The effect exerted by LOA occurs *via* the UPS given that the fatty acid-induced loss in SNAT2 can be attenuated by MG132, a potent cell permeable

proteasomal inhibitor. In contrast, the repressive effect of PA on SNAT2 protein/activity is likely to involve its derived lipid, ceramide, and possible SNAT2 degradaton by a non-UPS route.

The aims of the studies reported in this chapter were as follows:

- To investigate the effects of OA, LOA and PA upon the SNAT2 adaptation response to hypertonicity and/or amino acid withdrawal in skeletal muscle cells and HeLa cells.
- To investigate how LOA induces a loss in SNAT2 protein abundance in skeletal muscle cells and HeLa cells.
- To assess whether the repressive effect of PA upon SNAT2 activity is mediated by its derived lipid ceramide in skeletal muscle cells.

4.2 Results

4.2.1 Amino acid deprivation induces SNAT2 adaptation response in L6 myotubes

To assess System A adaptation to extracellular amino acid limitation in skeletal muscle cells, L6 myotubes were incubated with amino acid containing media for 24 h and amino acid deprived for the last 4 h of this incubation. As mentioned earlier, SNAT2 adaptation response consists of both an increase in SNAT2 transcription and enhanced SNAT2 protein stabilization. Fig 4.1A and 4.1B show that a 4 h amino acid deprivation period induces a ~2-fold increase in SNAT2 mRNA abundance and significantly enhances SNAT2 protein content in L6 myotubes. It is noteworthy that under amino acid restricted conditions, antibodies to SNAT2 detect two distinct but broad protein bands of ~60 kDa and ~40 kDa respectively, in L6 myotubes. Previous work from the Hundal lab has reported that the 60 kDa band most likely represents the “heavy” mature cell surface glycosylated transporter, whereas the diffuse “lighter” band most likely reflects a partially processed but immature intracellular pool of SNAT2 protein (Hyde et al., 2001). Consistent with the increase in SNAT2 protein, amino acid deprivation resulted in a ~6-fold increase in SNAT2 transport activity measured using Me-AIB, a paradigm non-metabolisable System A/SNAT2 substrate (Fig 4.1C).

4.2.2 Medium/long chain fatty acids repress the System A adaptation response

To assess whether System A adaptation to extracellular amino acid withdrawal would be sensitive to fatty acid availability I initially monitored the effect of different saturated, monunsaturated and polyunsaturated fatty acids upon

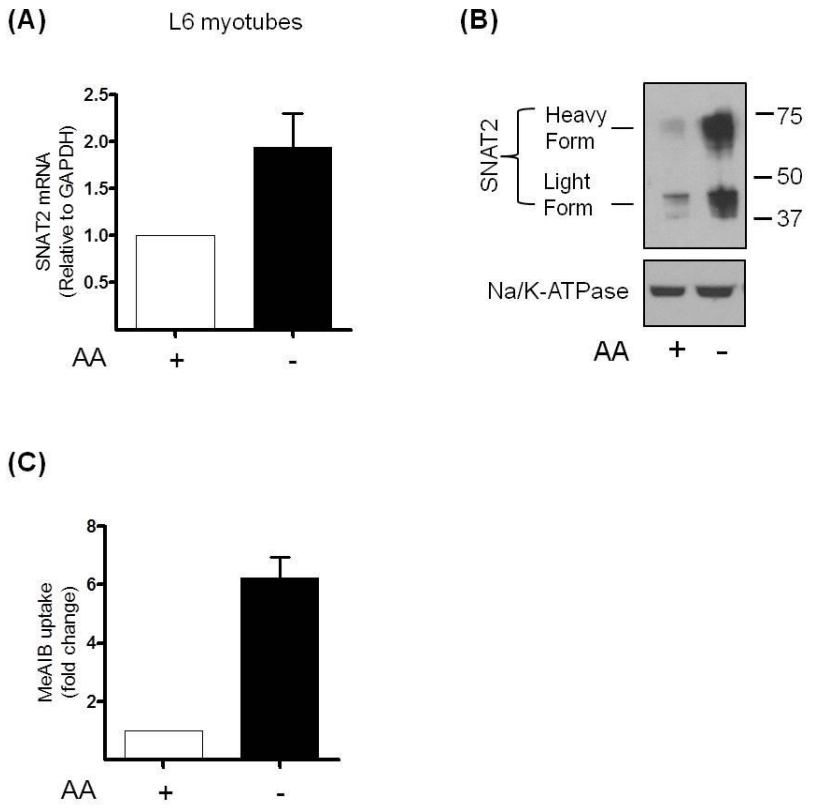


Figure 4.1 Amino acid deprivation induces SNAT2 adaptation response in L6 myotubes.

(A-C) L6 myotubes were incubated with EBSS media containing amino acids (+) for 24 h and amino acid deprived (-) for the last 4 h of this incubation. (A) The expression of SNAT2 was tested by quantitative PCR analysis of RNA isolated from L6 myotubes. (B) Total membranes were isolated and immunoblotted using antibodies against SNAT2 and Na/K-ATPase. (C) System A/SNAT2 activity was assayed by measuring the uptake of MeAIB. Data in the bar graphs is presented as mean ± SEM (n=3).

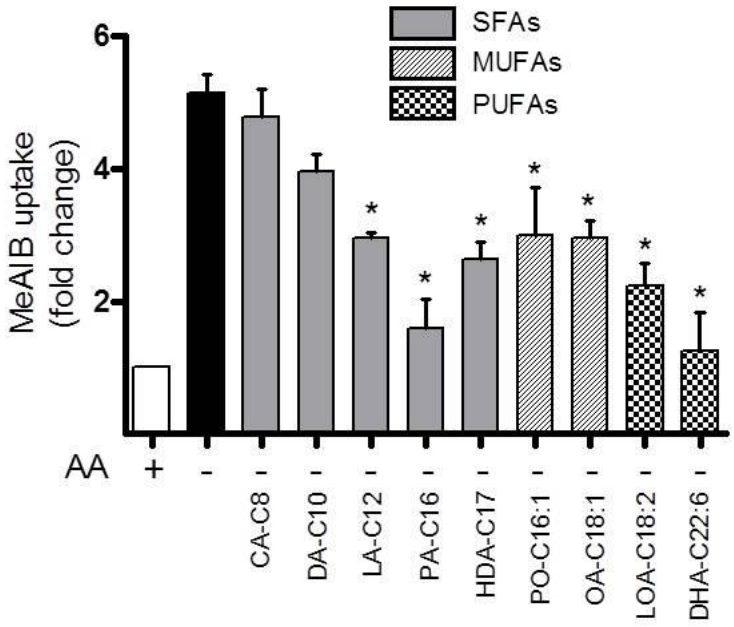


Figure 4.2 Long-chain fatty acids repress the adaptive increase in System A transport activity.

L6 myotubes were incubated with EBSS media containing amino acids (+) and 2% (w/v) BSA ± 300 μM caprylic acid (CA, C8), decanoic acid (DA, C10), lauric acid (LA, C12), palmitate (PA, C16), heptadecanoic acid (HAD, C17), palmitoleate (PO, C16:1 ω-7), oleic acid (OA, C18:1 ω-9), linoleic acid (LOA, C18:2 ω-6), docosahexanoic acid (DHA, C22:6 ω-3) for 24 h and amino acid deprived (-) for the last 4 h of this incubation as indicated prior to measuring MeAIB transport. The bar graph values are presented as mean ± SEM of three separate experiments each conducted in triplicate with asterisks indicating a significant change ($P<0.05$) from the solid black bar value.

System A transport activity assayed by analysis of Me-AIB uptake. Fig 4.2 shows that fatty acids with a carbon chain equal or longer than 12 carbons induce a decrease in System A adaptation response despite differences in their degree of saturation.

4.2.3 Linoleic acid represses the System A adaptation response

Given that in Chapter 3 I had assessed the effect of linoleic acid (LOA; a polyunsaturated (C18:2) ω-6 fatty acid) and oleic acid (OA; a monounsaturated (C18:1) ω9 fatty acid) upon insulin signalling in cultured skeletal muscle cells, I focussed on investigating the effect of these unsaturated fatty acids upon amino acid transport and transporter turnover. Fig 4.3A and 4.3B show that a 4 h amino acid deprivation period induces more than a 6-fold increase in System A transport in L6 myotubes compared to cells held in amino acid-containing media. Strikingly, this adaptive increase in System A transport was reduced when myotubes were preincubated with LOA or OA in time and dose-dependent manner – the suppressive effect of the fatty acid being maximal when myotubes were treated with LOA or OA for 24 h at a dose of 300 μM, with the PUFA having a more potent effect than the MUFA. The observed suppressive effect of LOA on System A was not restricted to L6 myotubes. Fig 4.3C shows that HeLa cells (an immortalised human adenocarcinoma cell line) also display a significant (9-fold) increase in System A adaptation upon being subjected to amino acid withdrawal, but that this was strongly repressed when cells were pretreated with LOA.

To assess whether LOA and OA could also affect other amino acid transporters, L6 myotubes and HeLa cells were treated with the fatty acids as indicated for 24

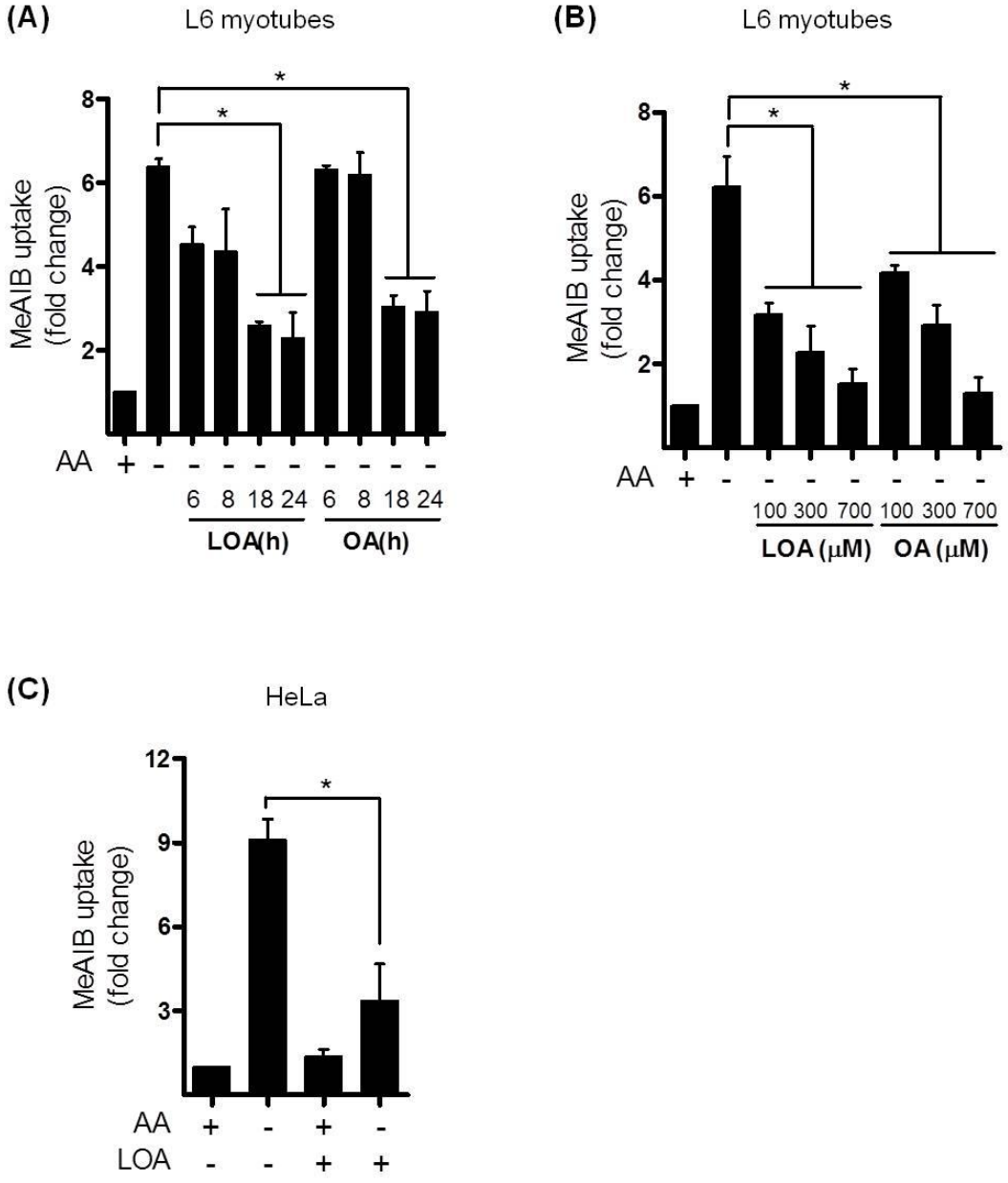

(A)
L6 myotubes
MeAIB uptake (fold change)
8
6
4
2
*
*
AA + - - - - - - - - - -
6 8 18 24 6 8 18 24
LOA(h)
OA(h)
(B)
L6 myotubes
MeAIB uptake (fold change)
8
6
4
2
*
*
AA + - - - - - - -
100 300 700 100 300 700
LOA (μM)
OA (μM)
(C)
HeLa
MeAIB uptake (fold change)
12
9
6
3
*
AA + - + -
LOA - - + +

Figure 4.3 The effect of LOA and OA on SystemA/SNAT2-mediated MeAIB uptake in L6 myotubes and HeLa cells.

(A) L6 myotubes were incubated with EBSS media containing amino acids (+) and 2% (w/v) BSA ± 300 μM linoleic acid (LOA) or oleic acid (OA) for times indicated and amino acid deprived (-) for the last 4 h of this incubation as indicated prior to measuring MeAIB transport. **(B)** L6 myotubes were incubated as in (A) ± increasing concentration of LOA or OA as indicated for 24 h and amino acid deprived (-) for the last 4 h of this incubation prior to measuring MeAIB transport. **(C)** HeLa cells were incubated as in (A) ± 300 μM linoleic acid (LOA) for 24 h and amino acid deprived (-) for the last 4 h of this incubation prior to measuring MeAIB transport. The bar graph values are presented as mean ± SEM of three separate experiments with asterisks indicating a significant change ($P<0.05$) between the indicated bars.

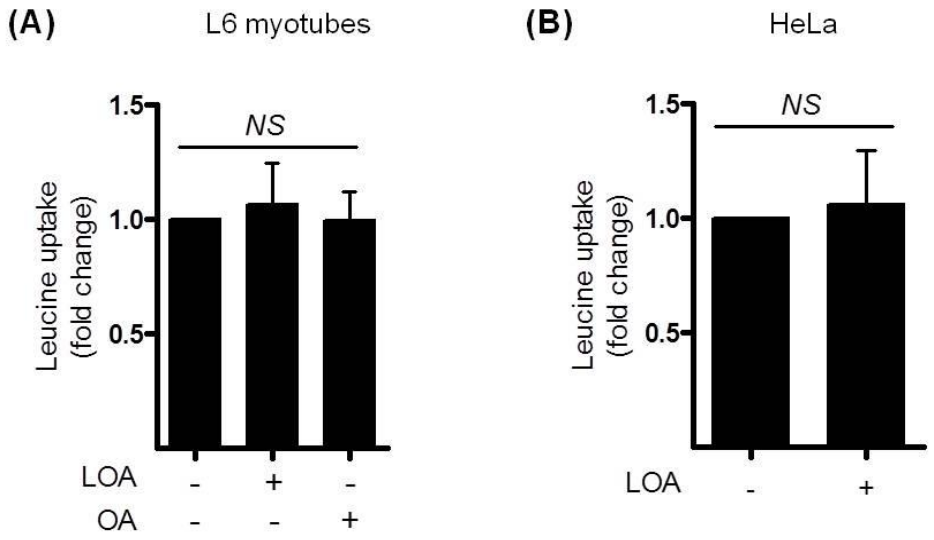


Figure 4.4 LOA and OA do not affect leucine uptake in L6 myotubes and HeLa cells.

(A, B) L6 myotubes (A) and HeLa cells (B) were incubated with EBSS media containing amino acids and 2% (w/v) BSA ± 300 μM linoleic acid (LOA) or oleic acid (OA) as indicated for 24 h prior to measuring leucine uptake. The bar graph values are presented as mean ± SEM of three separate experiments with *NS* indicating no significant change between the indicated bars.

h at a dose of 300 μM and leucine uptake assayed. Leucine transport is mediated by the System L family of transporters, a major nutrient transport system that is responsible for Na^+-independent transport of neutral amino acids (Hundal and Taylor, 2009). Fig 4.4A and 4.4B show that LOA or OA do not affect leucine uptake in L6 myotubes or HeLa cells indicating that provision of these unsaturated fatty acids may selectively target the SNAT2 transporter.

4.2.4 LOA impairs the adaptive increase in SNAT2 protein abundance

Previous studies from the Hundal lab have indicated that the adaptive response is principally attributed to increases in cellular SNAT2 abundance (Hyde et al., 2001). In line with this proposition, Fig 4.5A shows that there is a robust increase in protein abundance of both the mature and immature form of SNAT2 in total membranes prepared from L6 myotubes and HeLa cells following a 4h amino acid deprivation period. *In vitro* deglycosylation of total membranes with PNGaseF results in loss of the “heavier” immunoreactive band and detection of a more defined 40 kDa band that most likely represents the unprocessed core SNAT2 protein (Fig 4.5A left panel). My findings reveal that HeLa cells also display the adaptive increase in the mature 60 kDa SNAT2 protein, but, unlike L6 myotubes, exhibit very little expression of the “lighter” form of SNAT2. However, *in vitro* deglycosylation of the mature SNAT2 from HeLa cells also generates a more defined lighter 40 kDa SNAT2 band suggesting that HeLa cells readily process new synthesised SNAT2 and retain very little of the immature SNAT2 protein within intracellular membranes such as the ER (Fig 4.5A right panel). The adaptive increase in SNAT2 triggered by amino acid deprivation was substantially muted when L6 myotubes and HeLa cells were

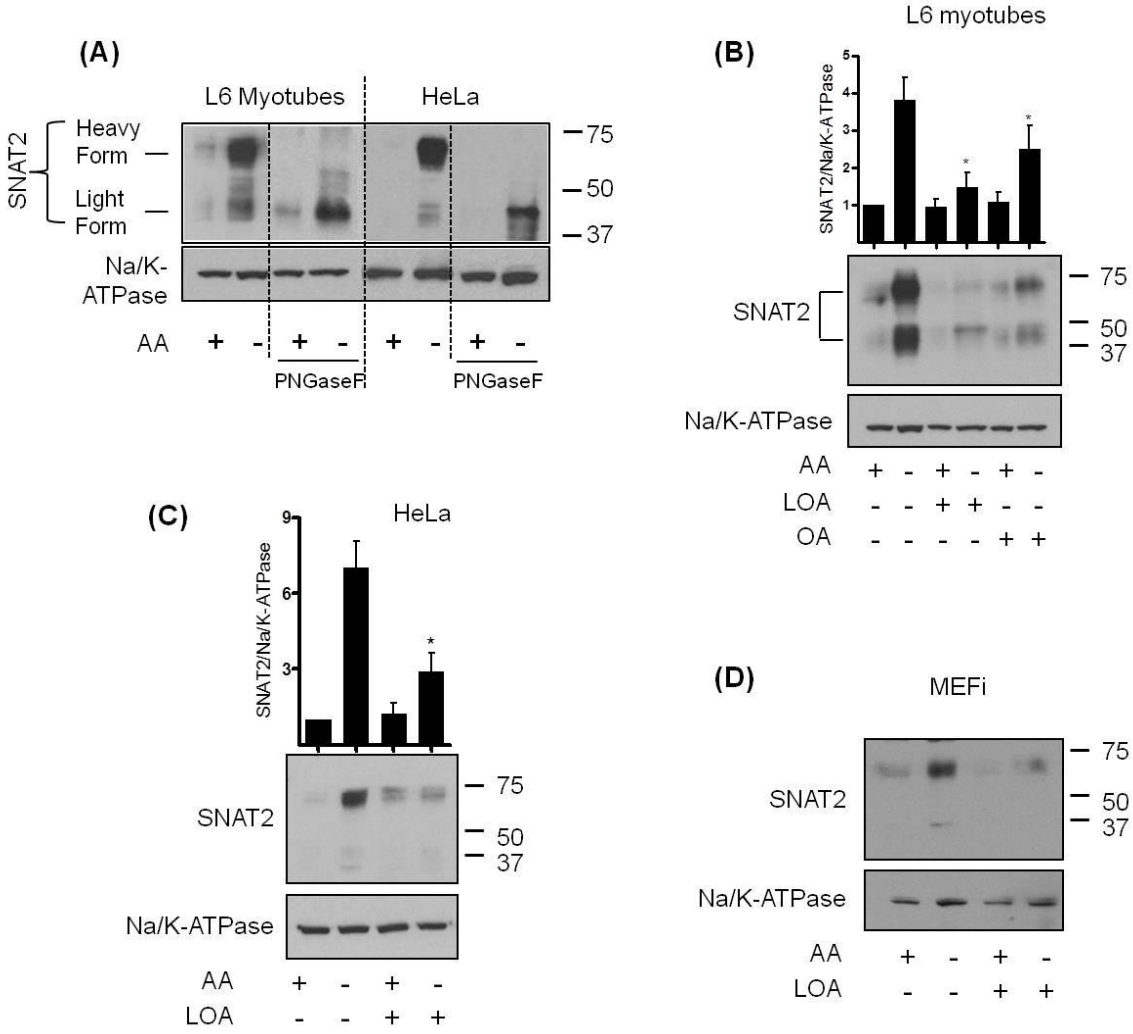

(A)
L6 Myotubes
HeLa
SNAT2
Heavy Form
Light Form
Na/K-ATPase
75
50
37
AA + - + - + - + -
PNGaseF
PNGaseF
(B)
L6 myotubes
SNAT2/Na/K-ATPase
SNAT2
Na/K-ATPase
75
50
37
AA + - + - + -
LOA - - + + - -
OA - - - - + +
(C)
HeLa
SNAT2/Na/K-ATPase
SNAT2
Na/K-ATPase
75
50
37
AA + - + -
LOA - - + +
(D)
MEFi
SNAT2
Na/K-ATPase
75
50
37
AA + - + -
LOA - - + +

Figure 4.5 LOA reduces SNAT2 protein abundance in response to amino acid deprivation in L6 myotubes, HeLa cells and MEFi cells.

(A-D) L6 myotubes, HeLa cells and MEFi cells were incubated with EBSS media containing amino acids (+) and 2% (w/v) BSA ± 300 μM linoleic acid (LOA) for 24 h and amino acid deprived (-) for the last 4 h of this incubation as indicated prior to total membranes isolation. In some experiments, membranes were deglycosylated using PNGaseF as indicated. Isolated total membranes from A-C were subsequently immunoblotted using antibodies against SNAT2 and Na/K-ATPase. The immunoblots are representative of three (A-C) and two (D) separate experiments. (B, C) Values in the bar graph are mean ± SEM (n=3) with asterisks signifying a significant change ($P<0.05$) compared to the amino acid deprived alone value.

pre-incubated with OA or LOA as indicated (Fig 4.5B and 4.5C), consistent with the diminished functional System A transport capacity that is observed in cells treated with the fatty acid (Fig 4.3). The repressive effect of LOA was still greater than that induced by OA and was not just restricted to L6 myotubes and HeLa cells but also observed in MEFi cells, an immortalised mouse embryonic fibroblast cell line (Fig 4.5D).

4.2.5 LOA does not suppress the transcriptional upregulation of SNAT2 gene expression

One possible explanation that may account for the reduction in SNAT2 adaptation induced by these unsaturated fatty acids is a failure to induce an upregulation in *SLC38A2* gene expression. However, quantitative PCR analysis of RNA isolated from L6 myotubes and HeLa cells revealed that OA and LOA do not significantly affect the increase in SNAT2 mRNA elicited in response to amino acid withdrawal (Fig 4.6A and 4.6B). The adaptive increase in SNAT2 gene expression is, in part, dependent upon ATF4 (Kilberg et al., 2005); a transcription factor whose expression, like that of the SNAT2 gene, is upregulated in response to amino acid deficiency. Fig 4.6C and 4.6D show that ATF4 expression is increased upon cellular amino acid depletion and that it remains unaffected by pre-incubation of cells with LOA or OA. This latter observation is fully in keeping with the increased transcription of the SNAT2 gene, an ATF4 target. Fig 4.6B and 4.6D show that HeLa cells do not exhibit a pronounced increase in SNAT2 and ATF4 gene expression in response to amino acid withdrawal as observed in L6 myotubes. Therefore, I wanted to assess whether enhanced SNAT2 trascription was, by comparison to L6

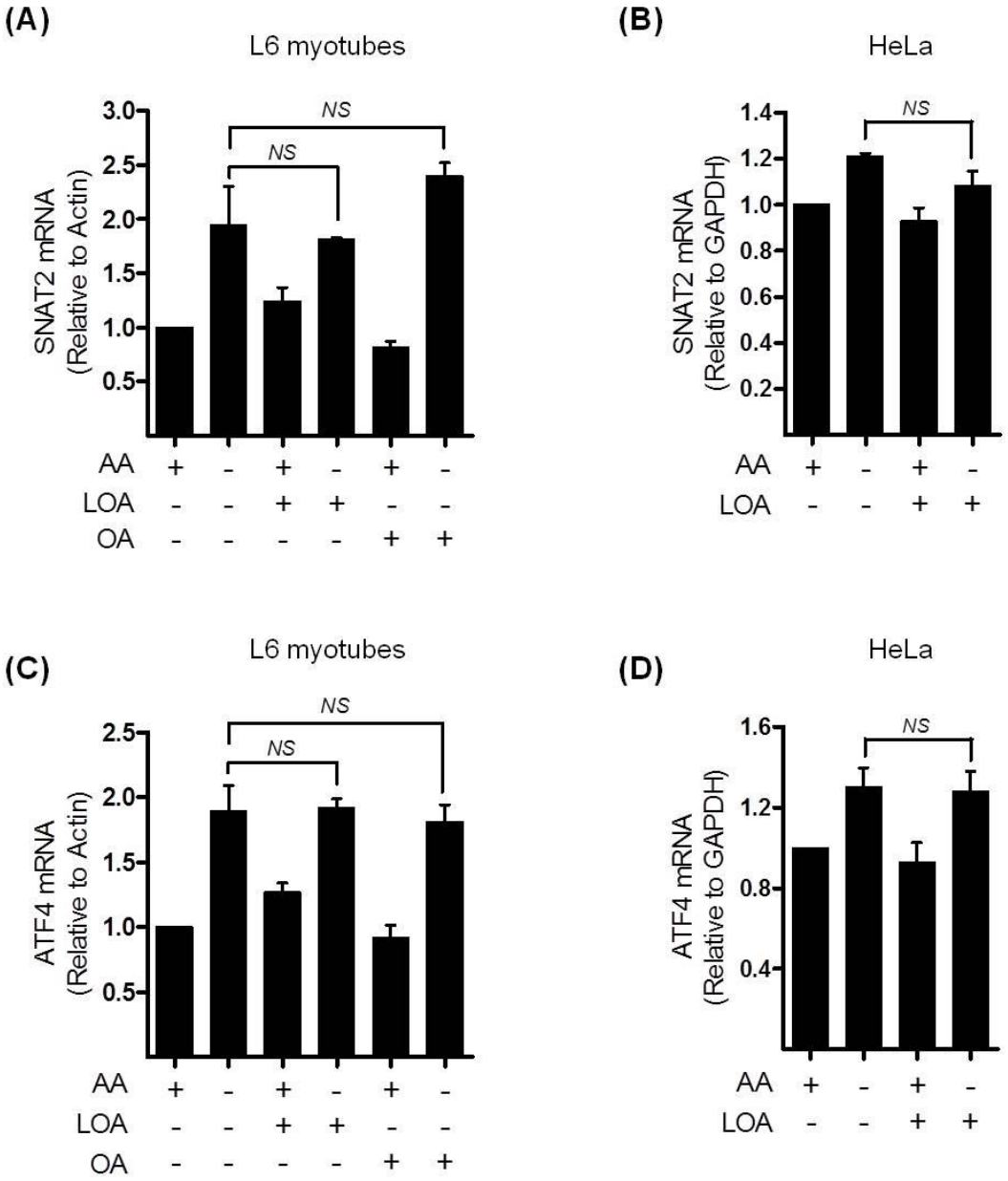

(A)
L6 myotubes
SNAT2 mRNA (Relative to Actin)
3.0
2.5
2.0
1.5
1.0
0.5
NS
NS
AA + - + - + -
LOA - - + + - -
OA - - - - + +
(B)
HeLa
SNAT2 mRNA (Relative to GAPDH)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
NS
AA + - + -
LOA - - + +
(C)
L6 myotubes
ATF4 mRNA (Relative to Actin)
2.5
2.0
1.5
1.0
0.5
NS
NS
AA + - + - + -
LOA - - + + - -
OA - - - - + +
(D)
HeLa
ATF4 mRNA (Relative to GAPDH)
1.6
1.2
0.8
0.4
NS
AA + - + -
LOA - - + +

Figure 4.6 LOA does not affect the transcriptional upregulation of SNAT2 in L6 myotubes and HeLa cells.

(A-D) L6 myotubes (A, C) and HeLa cells (B, D) were incubated with EBSS media containing amino acids (+) and 2% (w/v) BSA ± 300 µM linoleic acid (LOA) or oleic acid (OA) for 24 h and amino acid deprived (-) for the last 4 h of this incubation as indicated. The expression of SNAT2 (A, B) and ATF4 (C, D) was tested by quantitative PCR analysis of RNA isolated from L6 myotubes (A, C) and HeLa cells (B, D). Data in the bar graphs is presented as mean ± SEM (n=3) with *NS* indicating no significant change between the indicated bars.

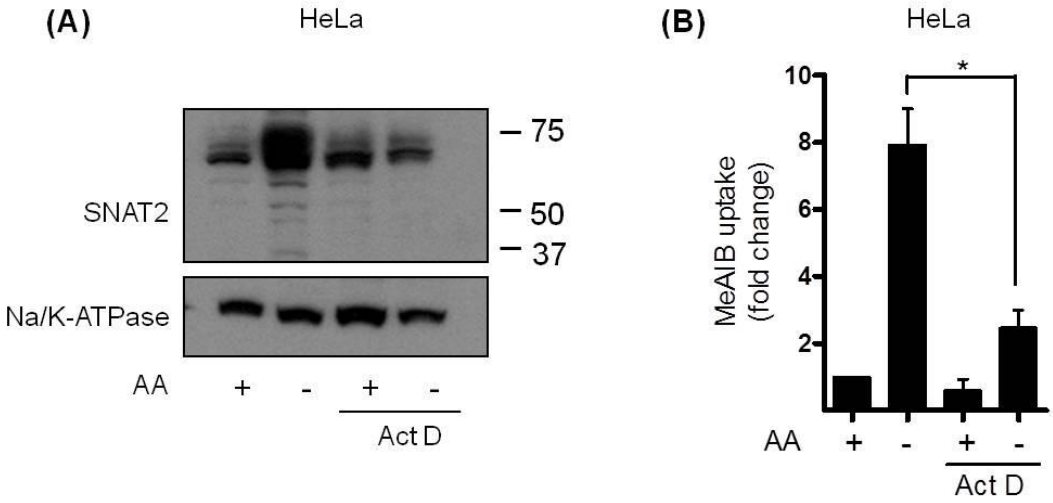


Figure 4.7 Increased SNAT2 transcription makes a major contribution to the System A adaptation response in HeLa cells.

(A, B) HeLa cells were incubated with EBSS media containing amino acids (+) for 24 h and amino acid deprived (-) for the last 4 h of this incubation. In some experiments cells were treated with Actinomycin D (5 μM) for the last 5 h of fatty acid incubation. (A) Total membranes were isolated and immunoblotted using antibodies against SNAT2 and Na/K-ATPase. The immunoblots are representative of three separate experiments. (B) System A/SNAT2 activity was assayed by measuring the uptake of MeAIB. Data in the bar graphs is presented as mean ± SEM (n=3) with asterisks indicating a significant change ($P<0.05$) between the bars indicated.

myotubes, a minor contributer to the SNAT2 adaptation response in this cell line. To address this issue, HeLa cells were incubated with amino acid containing media for 24 h and amino acid deprived for the last 4 h of this incubation in absence or presence of actinomycin D, a well established transcription inhibitor. Fig 4.7A and 4.7B show that the inhibitory effect of actinomycin D significantly reduced SNAT2 protein level and transport activity, suggesting that, despite the lower increase in SNAT2 and ATF4 mRNA, the adaptive response to amino acid withdrawal in HeLa cells is crucially dependent upon the modest increase in SNAT2 gene expression, which is likely to be efficiently translated.

4.2.6 LOA suppresses the adaptation response to hyperosmotic stress

System A expression/activity is also known to be transcriptionally upregulated in response to cellular hyperosmotic stress although the signaling mechanisms that drive this increase in SNAT2 gene expression are considered distinct from those that are responsive to amino acid withdrawal (Lopez-Fontanals et al., 2003).

Given that LOA exerted a potent suppressive effect on SNAT2/System A adaptation in response to amino acid starvation, I assessed if this fatty acid could also perturb the upregulation of SNAT2 expression/function normally induced by hyperosmotic stress. Fig 4.8A shows that L6 myotubes exposed to sucrose-induced hypertonic stress exhibit a 6-fold increase in SNAT2 mRNA expression and that this induction was unaffected in myotubes pretreated with LOA. However, subsequent analysis of System A function reveals that whilst System A-mediated Me-AIB uptake was elevated by ~4-fold in osmotically

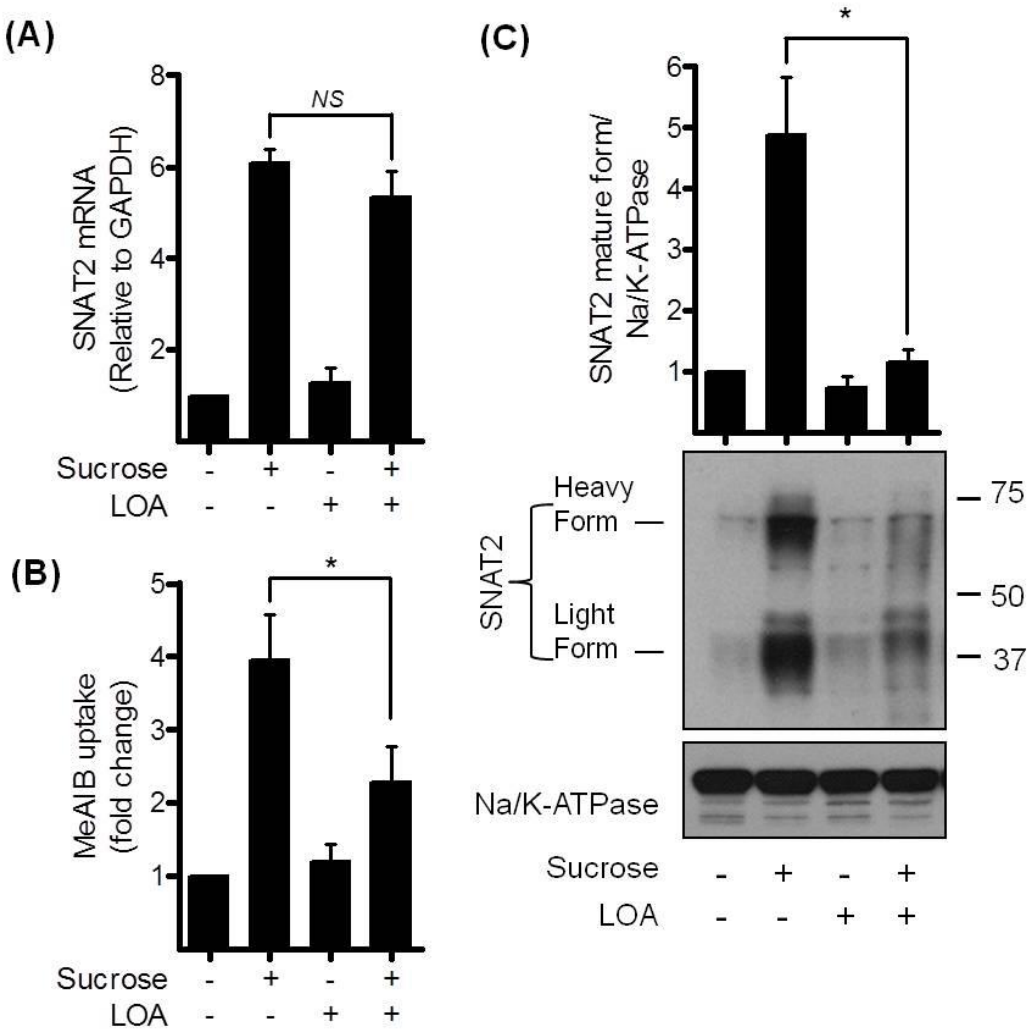

(A)
SNAT2 mRNA
(Relative to GAPDH)
NS
Sucrose - + - +
LOA - - + +
(B)
MeAIB uptake
(fold change)
*
Sucrose - + - +
LOA - - + +
(C)
SNAT2 mature form/
Na/K-ATPase
*
SNAT2
Heavy
Form
Light
Form
75
50
37
Na/K-ATPase
Sucrose - + - +
LOA - - + +

Figure 4.8 LOA suppresses the increase in SNAT2 expression/function induced in response to hyperosmotic stress in L6 myotubes.

(A-C) L6 myotubes were incubated with α-MEM and 2% (w/v) BSA ± 300 μM linoleic acid (LOA) for 24 h and with 100 mM sucrose for the last 4 h of this incubation as indicated. (A) The expression of SNAT2 was tested by quantitative PCR analysis of RNA isolated from L6 myotubes. (B) System A/SNAT2 activity was assayed by measuring the uptake of MeAIB. (C) Total membranes were isolated and immunoblotted using antibodies against SNAT2 and Na/K-ATPase. Data in the bar graphs is presented as mean ± SEM (n=3) with asterisks indicating a significant change ($P<0.05$) between the indicated pairs of bars.

shocked cells, it was significantly blunted in LOA-treated myotubes (Fig 4.8B). The observed loss in transport capacity tallies with loss of the mature (cell surface) and immature SNAT2 protein as judged by immunoblotting total membranes prepared from L6 myotubes with SNAT2 antibodies (Fig 4.8C).

4.2.7 Regulation of SNAT2 protein abundance by LOA

The data presented in Figure 4.5 support the idea that SNAT2 protein abundance may be specifically modulated in response to LOA availability in both L6 myotubes and HeLa cells. Since SNAT2 stability has previously been shown to be regulated by the ubiquitin proteasome system (UPS) (Hatanaka et al., 2006) I subsequently explored whether LOA may promote SNAT2 degradation *via* this proteolytic pathway. Consistent with the idea that SNAT2 ubiquitination/degradation would be reduced in amino acid deprived cells (*i.e.* those exhibiting an adaptive increase in SNAT2), HeLa cells transiently cotransfected with plasmids containing a CMV-driven SNAT2 construct (containing a C-terminal V5-His_6 epitope tag) and Flag-ubiquitin exhibit reduced SNAT2 ubiquitination compared to cells maintained in amino acid containing media under conditions when the UPS is inhibited with MG132 (Fig 4.9A). To assess the impact of LOA on cellular ubiquitination under amino acid deprived conditions whole cell lysates from HeLa cells incubated with the fatty acid for 24 h were immunoblotted with anti-ubiquitin antibodies. Fig 4.9B shows that the presence of MG132 is required for detection of accumulated ubiquitinated proteins and that HeLa cells incubated with LOA exhibit a significant increase in protein ubiquitination when the signal intensity is quantified along the vertical length of the gel lane using Image J software (Fig 4.9B upper panel). Intriguingly, whilst the expression of transiently expressed SNAT2-V5 in HeLa

cells under amino acid deprived conditions is not detected, the abundance of the V5-tagged transporter is considerably elevated upon inhibiting the UPS with MG132 (Fig 4.9C compare lanes 2 and 4). Importantly and in line with data already presented LOA induces a marked loss in SNAT2-V5 protein (compare lanes 2 and 3) that was not observed when MG132 was present (compare lanes 4 and 5).

The LOA-induced increase in protein ubiquitination is not restricted to HeLa cells. Fig 4.10A shows that, in the presence of MG132, L6 myotubes also exhibit the classic ubiquitin smear widely accepted as a typical biochemical signature for protein ubiquitination. The intensity of this ubiquitin smear was significantly enhanced in myotubes treated with LOA and, as expected, was further elevated with prolonged exposure to MG132 as this would contribute to increased accumulation of ubiquitinated proteins (Fig 4.10A). Interestingly, the increase in UPS and consequent protein breakdown promoted by LOA is likely to be accompanied by a decrease in protein synthesis, as indicated by reduced phosphorylation/activation of P70S6K1 and its downstream target S6, which serve as crucial regulators of mRNA translation (Fig 4.10B). Since LOA also attenuates increases in SNAT2 protein that occur in response to hypertonic stress it was not all together surprising to discover that the fatty acid also enhances protein ubiquitination in myotubes subjected to a 4 h hyperosmotic challenge with sucrose (Fig 4.10C). To further substantiate the idea that LOA can induce proteasomal degradation of cellular proteins, such as SNAT2, I monitored the effect of LOA on lysine 48 (K48) or 63 (K63)-specific ubiquitination using linkage-specific antibodies. K48-ubiquitin chains primarily target proteins for proteasomal degradation (Thrower et al., 2000), whereas

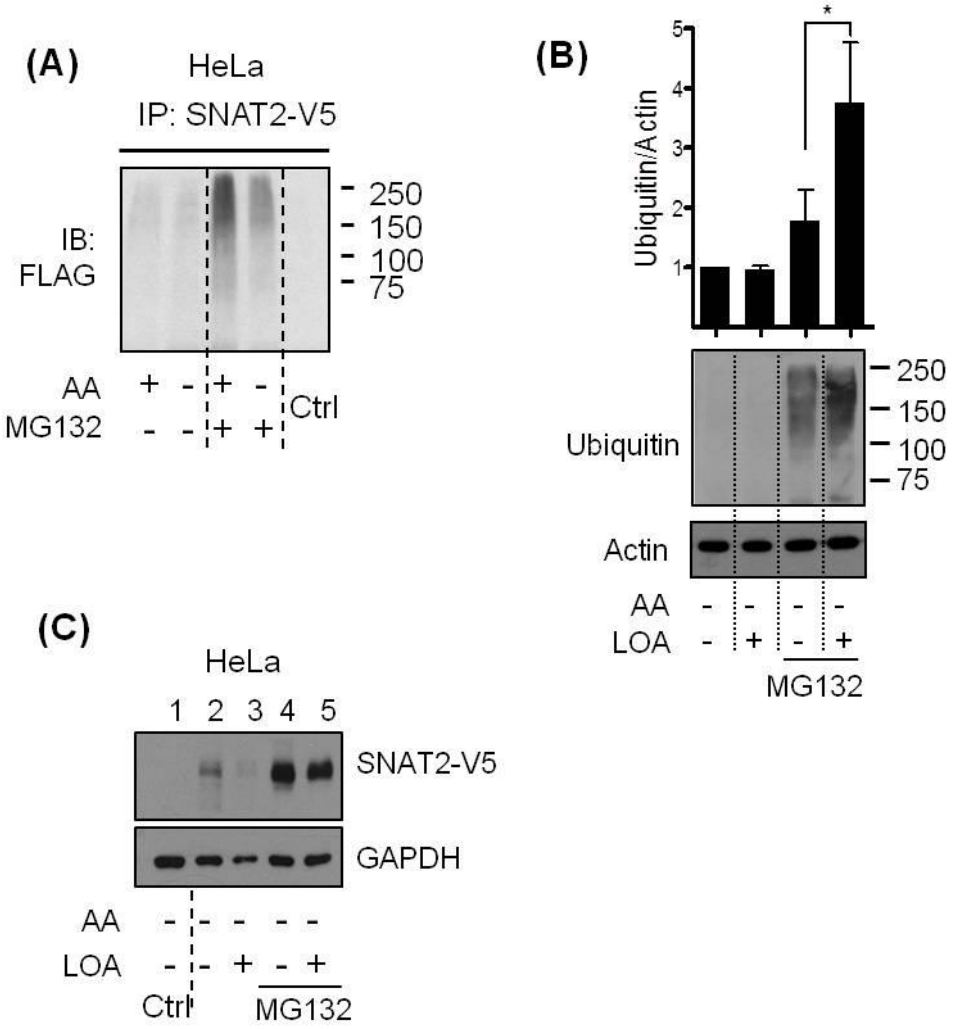

(A)
HeLa
IP: SNAT2-V5
IB:
FLAG
250
150
100
75
AA + - + -
MG132 - - + +
Ctrl
(B)
Ubiquitin/Actin
5
4
3
2
1
*
Ubiquitin
250
150
100
75
Actin
AA - - - -
LOA - + - +
MG132
(C)
HeLa
1 2 3 4 5
SNAT2-V5
GAPDH
AA - - - - -
LOA - - + - +
Ctrl
MG132

Figure 4.9 Proteasomal inhibition by MG132 prevents LOA-induced loss of SNAT2 protein.

(A) HeLa cells were transiently co-transfected with SNAT2-V5 and ubiquitin-Flag plasmids by the PEI method. 48 h post-transfection cells were incubated with EBSS media containing amino acids (+) for 8 h and amino acid deprived (-) for the last 4 h of this incubation in media containing or lacking MG132 (10 μM). SNAT2 was immunoprecipitated from whole cell lysates using an anti-V5 antibody and then immunoblotted using antibody against Flag to detect ubiquitin-Flag. **(B)** HeLa cells were incubated with EBSS media containing amino acids (+) and 2% (w/v) BSA ± 300 μM linoleic acid (LOA) for 24 h and amino acid deprived (-) for the last 4 h of this incubation in media containing or lacking MG132 (10 μM) as indicated. **(C)** HeLa cells were transiently transfected with SNAT2-V5 using lipofectamine. 24h post-transfection cells were incubated with EBSS media containing amino acids (+) and 2% (w/v) BSA ± 300 μM linoleic acid (LOA) for 24 h but amino acid deprived (-) during the last 4 h of this incubation in media containing or lacking MG132 (10 μM). Bar graph data is presented as mean ± SEM (n=3) with asterisks indicating a significant change ($P<0.05$) between the indicated bars.

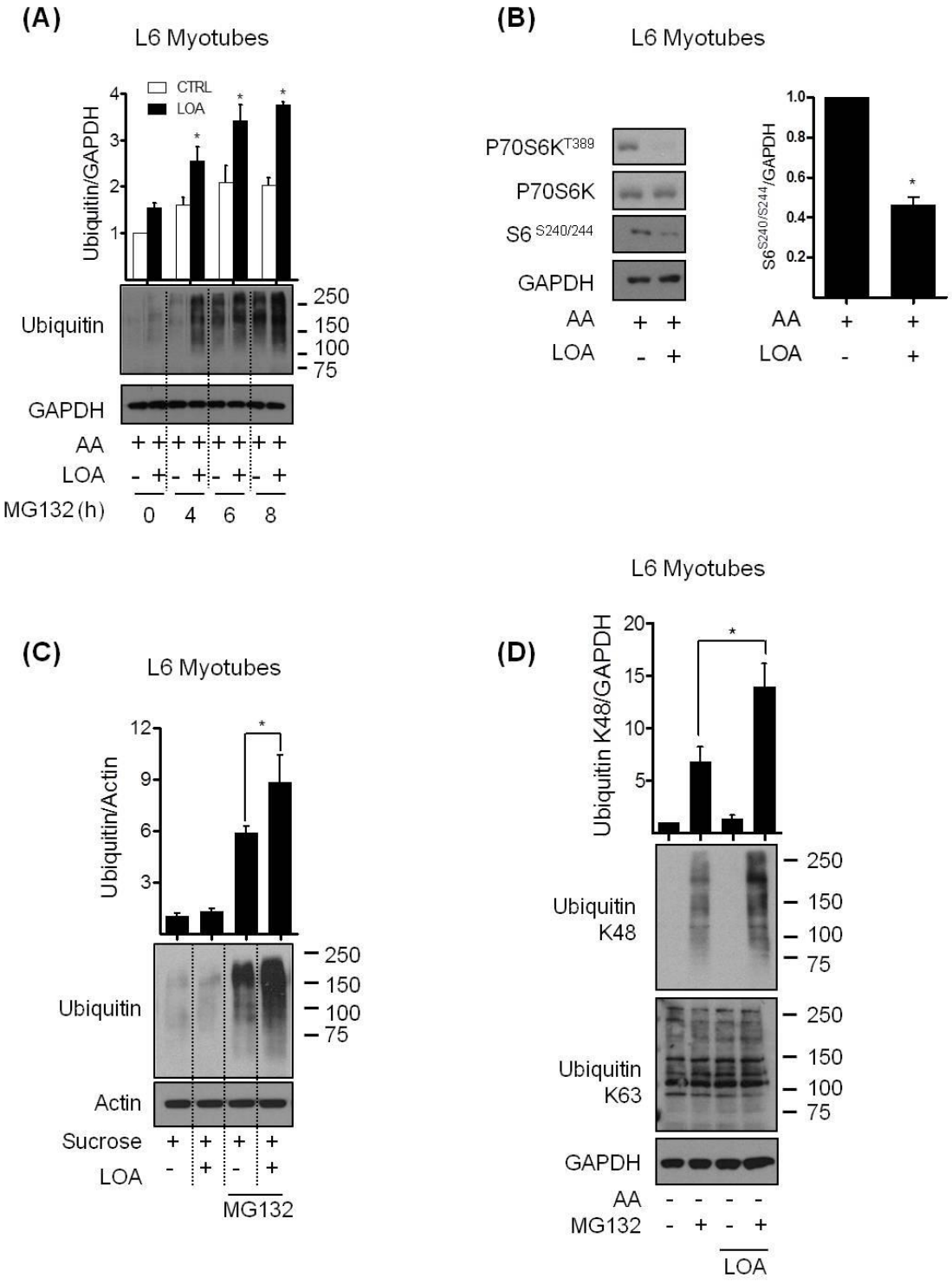

(A)
L6 Myotubes
CTRL
LOA
Ubiquitin/GAPDH
Ubiquitin
GAPDH
AA
LOA
MG132 (h)
0
4
6
8
250
150
100
75
(B)
L6 Myotubes
P70S6K^{T389}
P70S6K
S6 $^{S240/244}$
GAPDH
AA
LOA
S6$^{S240/S244}$/GAPDH
(C)
L6 Myotubes
Ubiquitin/Actin
Ubiquitin
Actin
Sucrose
LOA
MG132
(D)
L6 Myotubes
Ubiquitin K48/GAPDH
Ubiquitin K48
Ubiquitin K63
GAPDH
AA
MG132
LOA

Figure 4.10 LOA induces the UPS in L6 myotubes.

(A, B) L6 myotubes were incubated with EBSS media containing amino acids and 2% (w/v) BSA ± 300 µM linoleic acid (LOA) for 24 h. In some experiments cell were treated with MG132 (10 µM) for the penultimate hours of this incubation as indicated. **(C-D)** L6 myotubes were incubated with α-MEM (B) or EBSS (C) media containing amino acids and 2% (w/v) BSA ± 300 µM linoleic acid (LOA) for 24 h and treated with 100 mM sucrose (B) or amino acid deprived (C) for the last 4 h of this incubation in media containing or lacking MG132 (10 µM). Cell lysates from A-D were immunoblotted using antibodies against phospho/total proteins, ubiquitin or antibodies specific for detecting K48- and K63-linked ubiquitin as indicated. Immunoblots are representative of three distinct experiments. Data in the bar graphs is presented as mean ± SEM (n=3) with asterisks indicating a significant change ($P<0.05$) between the filled and unfilled bars (A), compared to control (B) or between the indicated bars (C, D).

formation of K63-ubiquitin chains direct proteins to alternative fates such as trafficking to the lysosome, participation in intracellular signalling and DNA repair in a manner unaffected by proteasomal inhibitors (Ikeda and Dikic, 2008). Fig 4.10D shows that whilst LOA induces a significant gain in cellular K48-protein ubiquitination I was unable to detect any notable changes in the K63-protein ubiquitination pattern irrespective of whether cells had been incubated with MG132 or not.

4.2.8 Effects of LOA and proteasomal inhibition on SNAT2 protein and System A transport

Having established that LOA can promote a net increase in cellular protein ubiquitination (Fig 4.10) and that MG132 can halt loss of SNAT2 protein by LOA (Fig 4.9C), I subsequently monitored the effect of LOA and MG132 on SNAT2 stability and function in L6 myotubes subjected to amino acid deprivation and hypertonic stress. Fig 4.11A and 4.11B show that a 4 h period of extracellular amino acid depletion or hypertonic stress induces a 4-5 fold increase in the expression of the mature “heavy” cell surface SNAT2 protein, which is associated with a significant (3-5 fold) increase in Me-AIB uptake under both circumstances (Fig 4.11C and 4.11D). However, pretreatment of L6 myotubes with LOA for 24 h prior to amino acid depletion or hypertonic stress during the penultimate 4 h of the LOA incubation period significantly restrains the increase in the abundance of both the “light” and “heavy” SNAT2 forms (Fig 4.11A and 4.11B) as well as the associated stimulus-induced increase in System A transport activity (Fig 4.11C and 4.11D). Intriguingly, cell treatment with MG132 not only halts the LOA-induced loss of the lighter SNAT2 form but induces its accumulation, whereas the inhibitor was unable to protect against loss of the

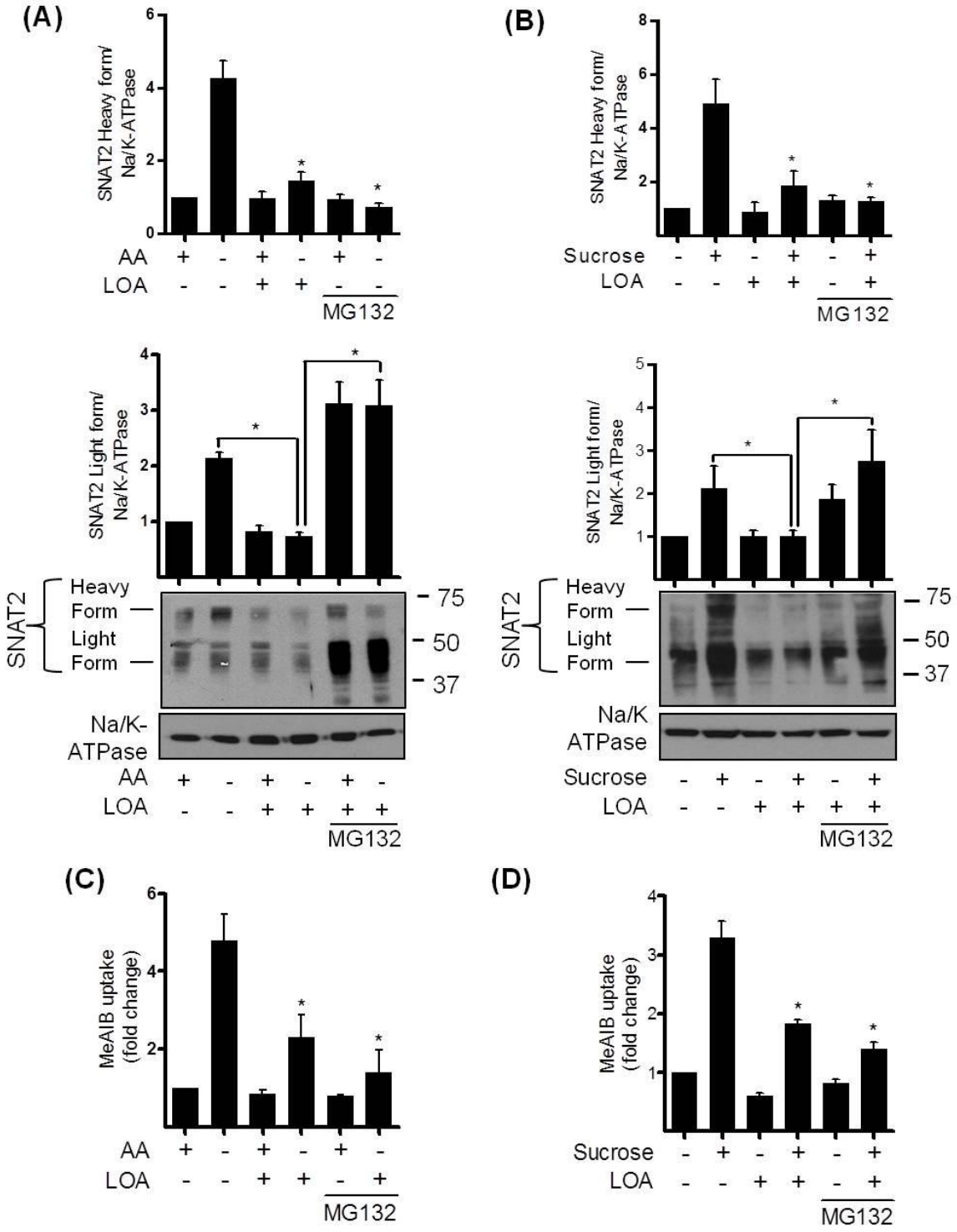

(A)
SNAT2 Heavy form/ Na/K-ATPase
AA
LOA
MG132
SNAT2 Light form/ Na/K-ATPase
SNAT2
Heavy Form
Light Form
Na/K-ATPase
75
50
37
(B)
Sucrose
(C)
MeAIB uptake (fold change)
(D)

Figure 4.11 LOA targets the immature SNAT2 form for proteasomal degradation in L6 myotubes subjected to amino acid withdrawal and hyperosmotic shock.

(A-D) L6 myotubes were incubated with EBSS media containing amino acids (A, C) or α-MEM (B, D) and 2% (w/v) BSA ± 300 μM linoleic acid (LOA) for 24 h and amino acid deprived (A, C) or stimulated with 100 mM sucrose (B, D) for the last 4 h of this incubation. (A, B) Total membranes were isolated and subsequently immunoblotted using antibodies against SNAT2 and Na/K-ATPase. (C, D) System A/SNAT2 activity was assayed by measuring the uptake of MeAIB. Data in the bar graphs is presented as mean ± SEM (n=3) with asterisks indicating a significant change ($P<0.05$) compared to the amino acid deprived (C) or the sucrose stimulated (D) alone value.

mature (cell surface) SNAT2 protein. The finding that Me-AIB uptake was not enhanced in cells that were amino acid depleted or those subject to hypertonic stress when having been pre-incubated with LOA and MG132 is fully consistent with loss of the cell surface SNAT2 protein.

4.2.9 Modulation of Nedd4.2 expression by LOA

One potential modifier of SNAT2 protein stability is Nedd4.2, an E3-ubiquitin ligase that has previously been implicated in the turnover of SNAT2 *via* the UPS (Hatanaka et al., 2006). Analysis of Nedd4.2 mRNA expression in both L6 myotubes and HeLa cells revealed that whilst amino acid availability *per se* had no effect on Nedd4.2 gene expression in presence of LOA, cells treated with LOA under amino acid deprived conditions exhibit a significant increase in both Nedd4.2 mRNA and protein abundance (Fig 4.12A-D). To further investigate if this LOA-induced increase in Nedd4.2 contributes to the destabilization/functional loss of SNAT2, expression of Nedd4.2 was stably silenced using shRNA in HeLa cells. Fig 4.13A shows that compared to control cells transfected with the empty lentiviral vector, Nedd4.2 protein was undetectable in cells expressing shRNA targeting Nedd4.2 mRNA. However, despite the effective silencing of Nedd4.2 expression, little impact was observed upon the adaptive increase in SNAT2 that occurs following amino acid withdrawal or upon the ability of LOA to curb the increase in both SNAT2 protein (Fig 4.13B) and System A transport activity (Fig 4.13C).

Work from the Hundal lab has previously indicated that the cytoplasmic N-terminal domain of SNAT2 plays an important role with respect to stability of the transporter (Hyde et al., 2007). Whilst the ubiquitination sites on SNAT2 have

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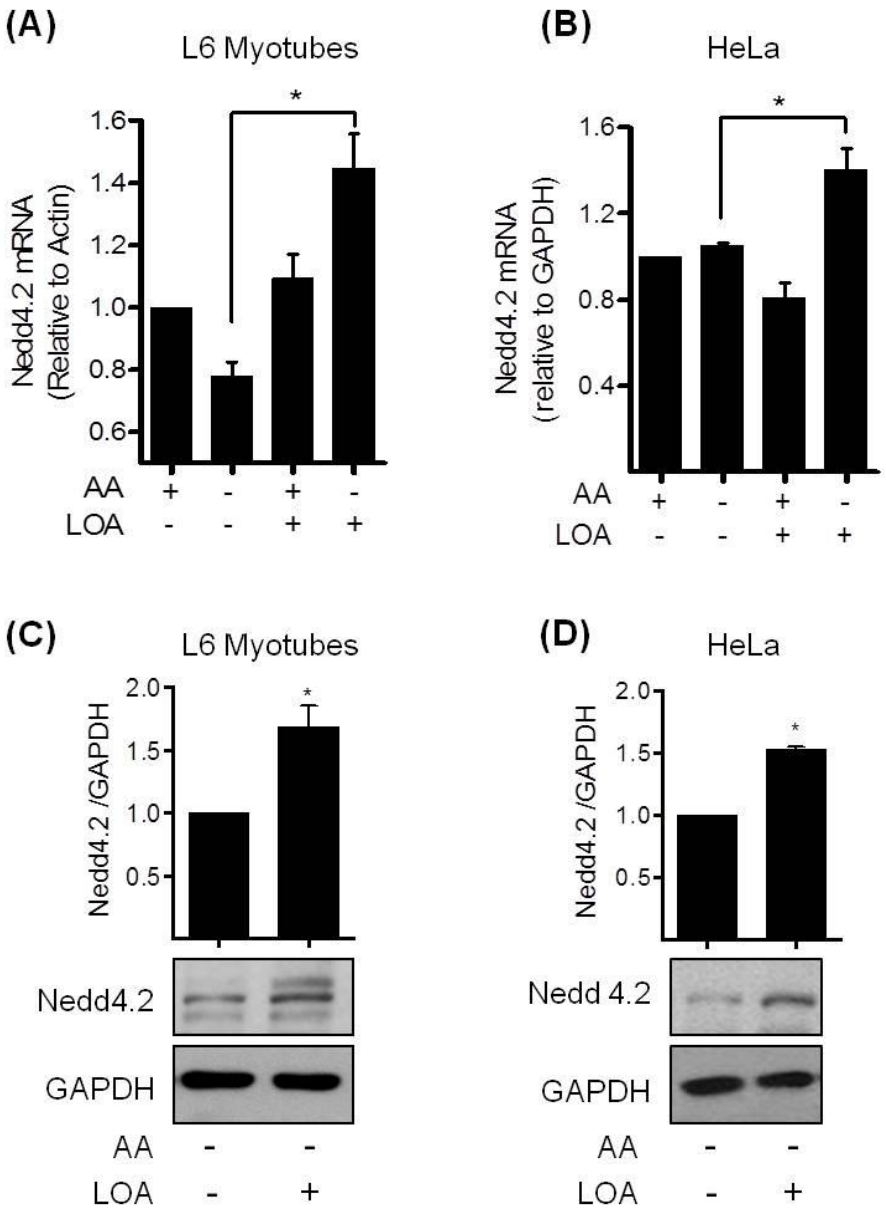

(A)
L6 Myotubes
Nedd4.2 mRNA (Relative to Actin)
1.6
1.4
1.2
1.0
0.8
0.6
*
AA + - + -
LOA - - + +
(B)
HeLa
Nedd4.2 mRNA (relative to GAPDH)
1.6
1.2
0.8
0.4
*
AA + - + -
LOA - - + +
(C)
L6 Myotubes
Nedd4.2 /GAPDH
2.0
1.5
1.0
0.5
*
Nedd4.2
GAPDH
AA - -
LOA - +
(D)
HeLa
Nedd4.2 /GAPDH
2.0
1.5
1.0
0.5
*
Nedd 4.2
GAPDH
AA - -
LOA - +

Figure 4.12 LOA enhances Nedd4.2 mRNA and protein level in amino acid-deprived L6 myotubes and HeLa cells.

(A-D) L6 myotubes (A, C) and HeLa cells (B, D) were incubated with EBSS media containing amino acids (+) and 2% (w/v) BSA ± 300 µM linoleic acid (LOA) for 24 h and amino acid deprived (-) for the last 4 h of this incubation as indicated. (A, B) The expression of Nedd4.2 was assessed by quantitative PCR analysis of RNA isolated from L6 myotubes (A) and HeLa cells (B). (C, D) Cell lysates were immunoblotted using antibodies against Nedd4.2 and GAPDH. Data in the bar graphs is presented as mean ± SEM (n=3) with asterisks indicating a significant change ($p<0.05$) between the indicated bars (A, B) or compared to the control (C, D).

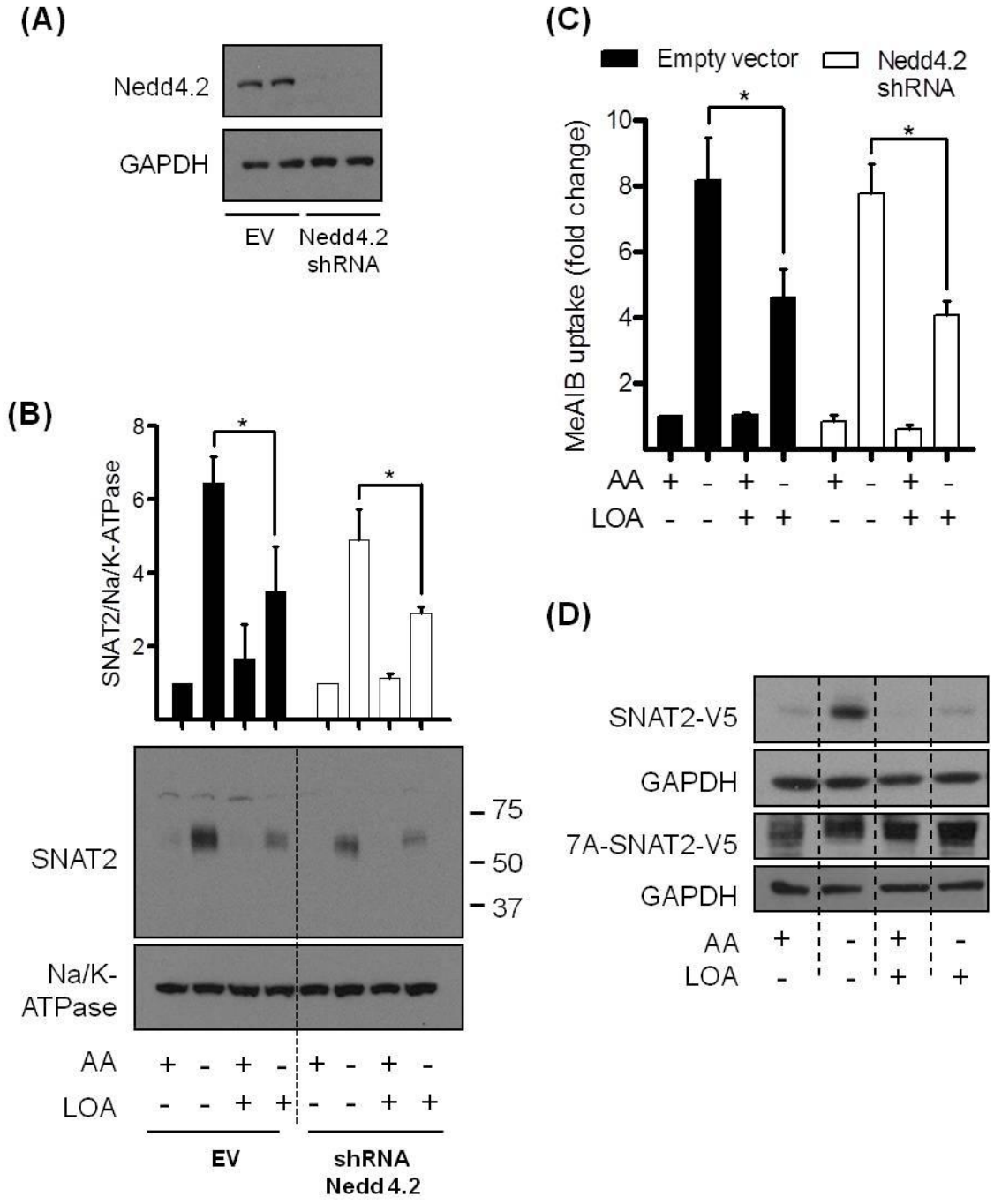

(A)
Nedd4.2
GAPDH
EV
Nedd4.2 shRNA
(B)
SNAT2/Na/K-ATPase
8
6
4
2
*
*
SNAT2
Na/K-ATPase
75
50
37
AA + - + - + - + -
LOA - - + + - - + +
EV
shRNA Nedd4.2
(C)
Empty vector
Nedd4.2 shRNA
MeAIB uptake (fold change)
10
8
6
4
2
*
*
AA + - + - + - + -
LOA - - + + - - + +
(D)
SNAT2-V5
GAPDH
7A-SNAT2-V5
GAPDH
AA + - + -
LOA - - + +

Figure 4.13 The suppressive effect of LOA upon SNAT2 adaptation does not involve Nedd4.2 in HeLa cells.

(A-C) HeLa cells stably expressing empty vector or shRNA targeting Nedd4.2 were incubated with EBSS media containing amino acids (+) and 2% (w/v) BSA ± 300 µM linoleic acid (LOA) for 24 h and amino acid deprived (-) for the last 4 h of this incubation as indicated. (A, B) Cell lysates (A) and isolated total membranes (B) were immunoblotted using antibodies against the proteins indicated. (C) System A/SNAT2 activity was assayed by measuring the uptake of MeAIB. **(D)** HeLa cells were transiently transfected with SNAT2-V5 or 7A-SNAT2-V5 mutant using lipofectamine. 24 h post-transfection cells were incubated with EBSS media containing amino acids (+) and 2% (w/v) BSA ± 300 µM linoleic acid (LOA) for 24 h and amino acid deprived (-) for the last 4 h of this incubation. Cell lysates were immunoblotted using antibodies against V5 and GAPDH. Immunoblots are representative of three (A, B) and two (D) distinct experiments. Data in the bar graphs is presented as mean ± SEM (n=3) with asterisks indicating a significant change ($P<0.05$) between the indicated bars (B, C).

not yet been mapped, of the 12 cytosolic exposed lysine residues that may potentially be ubiquitinated, seven are located within the N-terminal tail. Thus, one or more of these lysine residues may influence SNAT2 stability. To test this possibility all 7 lysine residues in the N-terminal tail were mutated to an alanine (7A mutant) and the mutated 7A-SNAT2-V5 construct expressed from a CMV-non amino acid responsive promoter. The reason for using this strategy was to avoid increases in SNAT2 transcription driven by amino acid deprivation and allow focus on SNAT2 protein stability alone. Figure 4.13D shows that whilst HeLa cells expressing wild-type SNAT2-V5 only exhibit stabilization of SNAT2 protein in amino acid deprived cells, the 7A-SNAT2-V5 appears to be equally stable under all conditions tested.

4.2.10 Effects of palmitate upon the System A adaptation response in L6 myotubes

Interestingly, whilst palmitate (PA) and LOA are known to have opposing effects upon insulin signalling, both induced a similar repressive effect on SNAT2 transport activity (Fig 4.2). To assess whether PA and LOA promote this repression by the same mechanism I further investigated the effect of PA provision upon SNAT2 transport activity, protein abundance and mRNA level in L6 myotubes and HeLa cells. Previous work from the Hundal lab has already demonstrated that PA represses basal System A transport activity in L6 myotubes (Fig 4.14A). Fig 4.14B and 4.14C show that the fatty acid reduces also the adaptive increase in System A transport in a time- and dose-dependent manner in this skeletal muscle cell line. Interestingly, unlike LOA, the suppressive effect of PA upon System A adaptation was not observed in HeLa

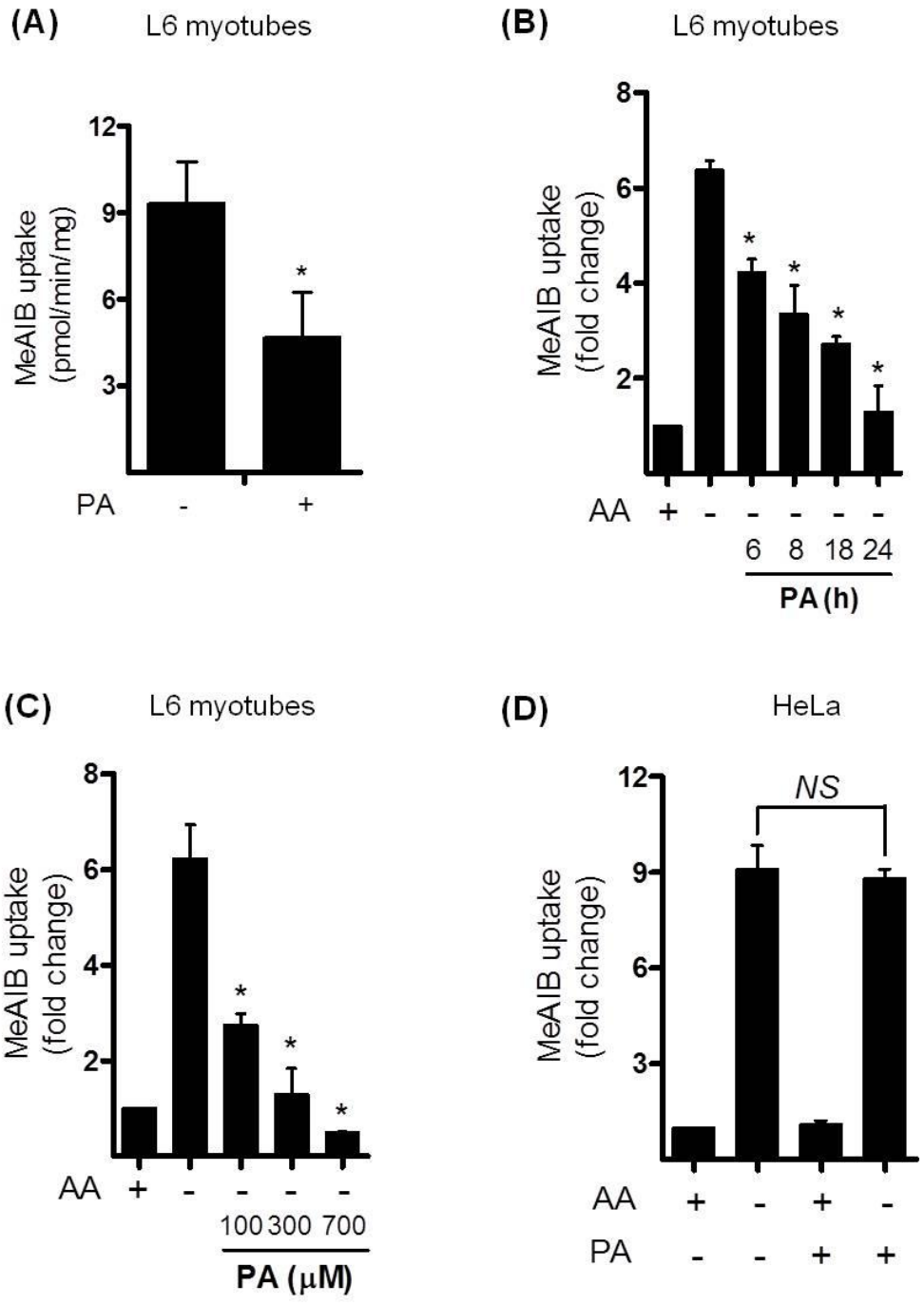

(A)
L6 myotubes
MeAIB uptake (pmol/min/mg)
12
9
6
3
*
PA - +
(B)
L6 myotubes
MeAIB uptake (fold change)
8
6
4
2
* * * *
AA + - - - - -
6 8 18 24
PA (h)
(C)
L6 myotubes
MeAIB uptake (fold change)
8
6
4
2
* * *
AA + - - - -
100 300 700
PA (μM)
(D)
HeLa
MeAIB uptake (fold change)
12
9
6
3
NS
AA + - + -
PA - - + +

Figure 4.14 Effects of PA on SystemA/SNAT2-mediated Me-AIB uptake in L6 myotubes and HeLa cells.

(A) L6 myotubes were incubated with EBSS media containing amino acids (+) and 2% (w/v) BSA ± 750 μM palmitate (PA) for 16 h prior to measuring MeAIB transport. **(B)** L6 myotubes were incubated with EBSS media containing amino acids (+) and 2% (w/v) BSA ± 300 μM palmitate (PA) for times indicated and amino acid deprived (-) for the last 4 h of this incubation prior to measuring MeAIB transport. **(C)** L6 myotubes were incubated as in (A) ± increasing concentration of PA as indicated for 24 h and amino acid deprived (-) for the last 4 h of this incubation prior to measuring MeAIB transport. **(D)** HeLa cells were incubated as in (A) ± 300 μM palmitate (PA) for 24 h and amino acid deprived for the last 4 h of this incubation prior to measuring MeAIB transport. The bar graph values are presented as mean ± SEM of three separate experiments with asterisks indicating a significant change ($P<0.05$) compared to the amino acid deprived alone value and *NS* no significant change between the indicated bars.

cells indicating that the fatty acid may have a cell-specific effect on the modulation of the SNAT2 adaptive response (Fig 4.14D).

4.2.11 PA impairs abundance of the mature glycosylated SNAT2 without affecting that of the immature form in L6 myotubes

To assess whether the observed reduction in System A transport induced by PA could be attributed to changes in SNAT2 protein abundance, L6 myotubes and HeLa cells were incubated with 300 μM PA in medium containing amino acids and amino acid deprived for the last 4 h of this incubation before total membranes were isolated. Fig 4.15A shows that the protein abundance of both the glycosylated mature form and immature intracellular form of SNAT2 was significantly increased by amino acid withdrawal in L6 myotubes. The adaptive increase in the mature “heavy” SNAT2 form was reduced substantially when L6 myotubes were incubated with PA (Fig 4.15.A), in line with the reduction in System A transport activity induced by the saturated fatty acid (Fig 4.14B and 4.14C). PA treatment results also in a slight reduction of mature SNAT2 protein abundance in cells held in amino acid containing media (Fig 4.15A, compare lanes 1 and 3), consistent with PA-induced reduction of basal System A transport acitivty (Fig 4.14A). However, unlike LOA (Fig 4.5B), PA provision does not affect the abundance of the immature intracellular form of the transporter, which is still enhanced to a level comparable to amino acid deprived cells not exposed to the fatty acid (Fig 4.15A). Moreovoer, PA does not affect the adaptive increase in SNAT2 protein abundance in HeLa cells (Fig 4.15B), consistent with the finding that it also has no effect on System A transport activity in this cell line (Fig 4.14D).

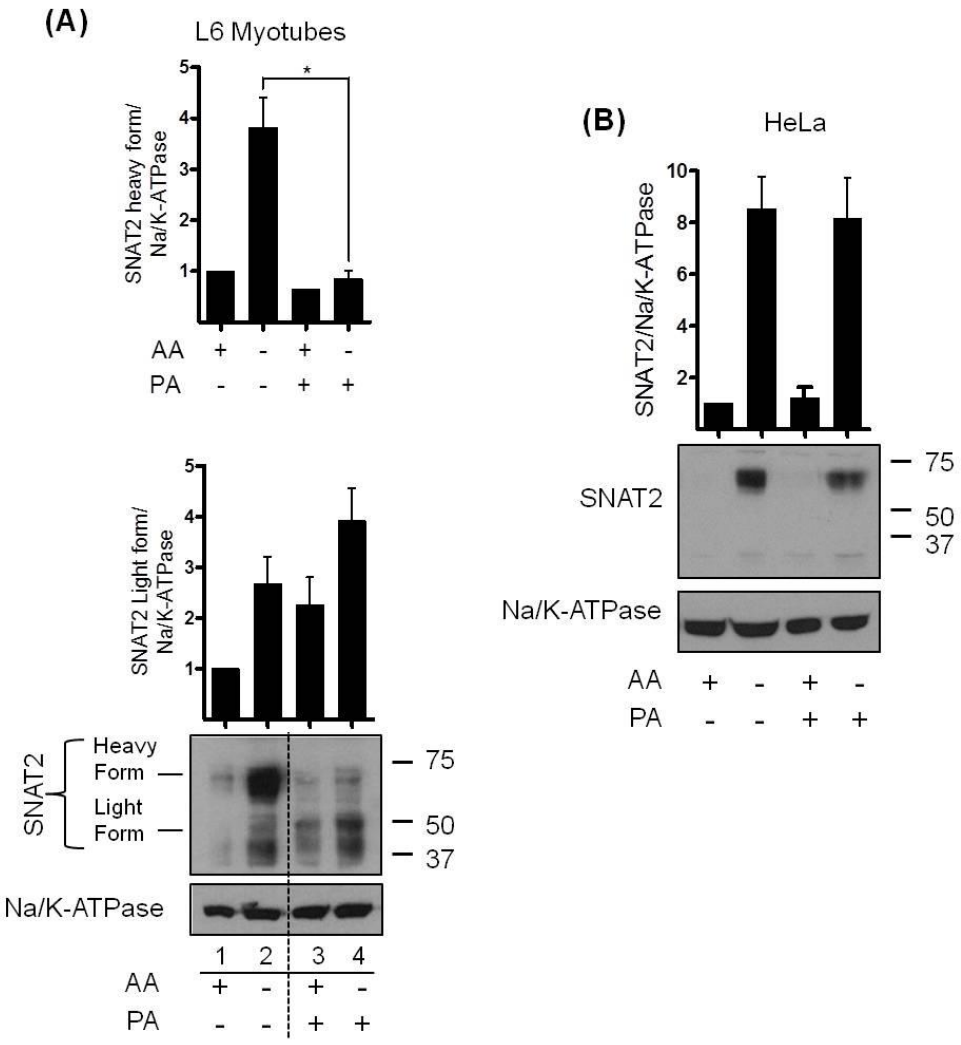


Figure 4.15 Effects of PA upon SNAT2 protein abundance in L6 myotubes but not in HeLa cells.

(A, B) L6 myotubes and HeLa cells were incubated with EBSS media containing amino acids (+) and 2% (w/v) BSA ± 300 μM palmitate (PA) for 24 h and amino acid deprived (-) for the last 4 h of this incubation as indicated prior to isolation of total membranes. Total membranes were subsequently immunoblotted using antibodies against SNAT2 and Na/K-ATPase. The immunoblots are representative of three separate experiments. The asterisks signify a significant change ($P<0.05$) between the indicated bars.

4.2.12 Effects of PA upon SNAT2 gene transcription in L6 myotubes

As mentioned earlier, fatty acids may potentially reduce the SNAT2 adaptive response *via* impaired upregulation of the *SLC38A2* gene. Surprisingly, however, Fig 4.16A shows that SNAT2 mRNA abundance was increased in L6 myotubes pre-incubated with PA regardless of whether cells were subjected to amino acid deprivation or not. This observation is consistent with the finding that ATF4 mRNA abundance is also enhanced by PA in L6 myotubes held in amino acid containing media (Fig 4.16C). In contrast, and in line with the findings that PA has no effect upon SNAT2 protein abundance and transport activity in response to amino acid withdrawal in HeLa cells, also the adaptive increase in mRNA abundance of SNAT2 and ATF4 of cells pre-incubated with the saturated fatty acid are comparable to that of amino acid deprived cells not exposed to PA (Fig 4.16B and 4.16D).

It is well documented that PA can induce pro-inflammatory signalling and ER stress in insulin target tissues (Green et al., 2011; Macrae et al., 2013; Salvado et al., 2013). ATF4 is one of several transcription factors activated by ER-stress and PA has been shown to increase ATF4 mRNA abundance in hepatocytes (Wei et al., 2009; Wei et al., 2006), which is in line with my findings in skeletal muscle cells (Fig 4.16C). Since OA has been shown to prevent PA-induced ER stress (Salvado et al., 2013), I wanted to test whether co-provision of the MUFA could counteract the increase in SNAT2 and ATF4 mRNA level triggered by PA in L6 myotubes. Fig 4.17A and 4.17B show that, while OA *per se* does not affect SNAT2 and ATF4 mRNA expression, PA induces a ~2-fold increase. However, when PA is co-applied with OA, the MUFA antagonises the

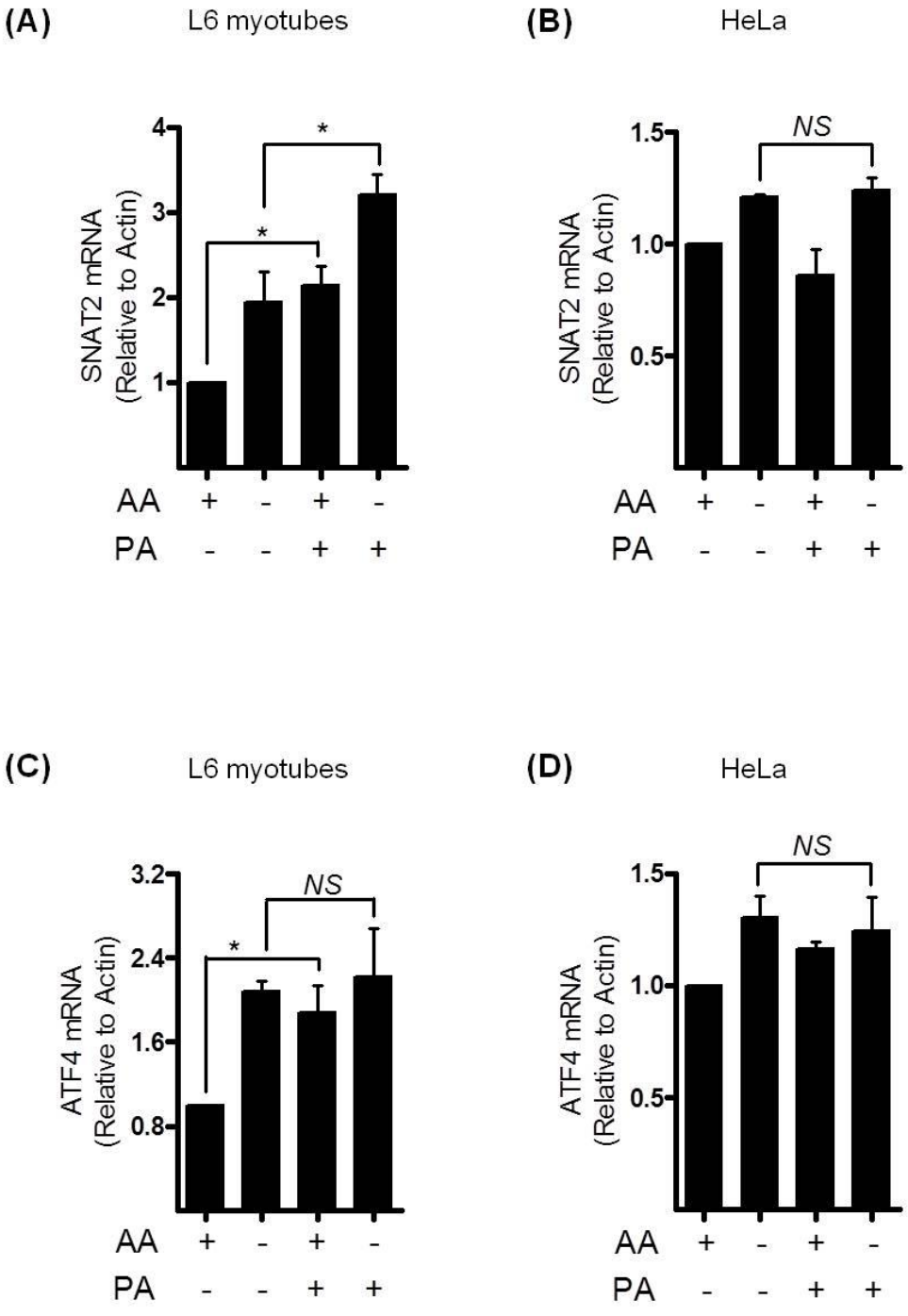

(A)
L6 myotubes
SNAT2 mRNA (Relative to Actin)
4
3
2
1
*
*
AA + - + -
PA - - + +
(B)
HeLa
SNAT2 mRNA (Relative to Actin)
1.5
1.0
0.5
NS
AA + - + -
PA - - + +
(C)
L6 myotubes
ATF4 mRNA (Relative to Actin)
3.2
2.4
1.6
0.8
*
NS
AA + - + -
PA - - + +
(D)
HeLa
ATF4 mRNA (Relative to Actin)
1.5
1.0
0.5
NS
AA + - + -
PA - - + +

Figure 4.16 PA does not repress the transcriptional upregulation of SNAT2 in L6 myotubes and HeLa cells.

(A-D) L6 myotubes (A, C) and HeLa cells (B, D) were incubated with EBSS media containing amino acids (+) and 2% (w/v) BSA ± 300 µM palmitate (PA) for 24 h and amino acid deprived (-) for the last 4 h of this incubation as indicated. The expression of SNAT2 (A, B) and ATF4 (C, D) was tested by quantitative PCR analysis of RNA isolated from L6 myotubes (A, C) and HeLa cells (B, D). Data in the bar graphs is presented as mean ± SEM (n=3) with asterisks indicating a significant difference ($p<0.05$) and *NS* a no significant change between the indicated bars.

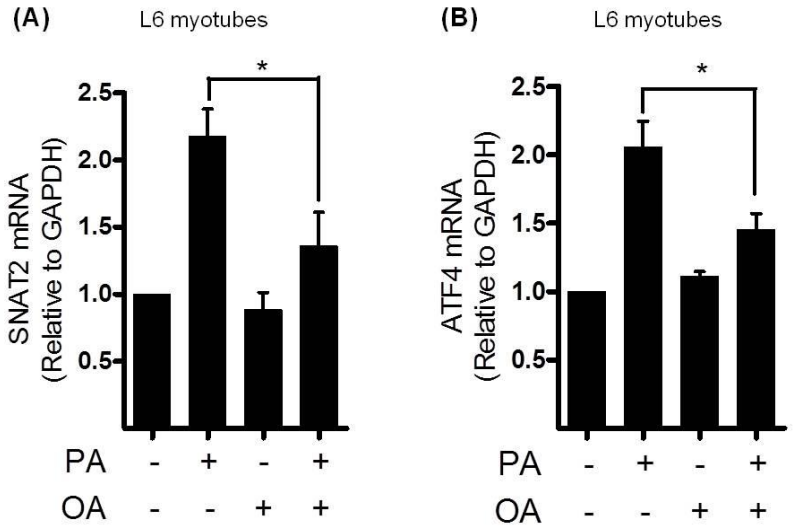


Figure 4.17 OA prevents the activatory effect of PA on SNAT2 transcription in L6 myotubes.

(A, B) L6 myotubes were incubated with EBSS media containing amino acids and 2% BSA ± 300 µM palmitate (PA), oleic acid (OA) or both for 24 h as indicated. The expression of SNAT2 (A) and ATF4 (B) was tested by quantitative PCR analysis of RNA isolated from L6 myotubes. Data in the bar graphs is presented as mean ± SEM (n=3) with asterisk indicating a significant difference ($p<0.05$) between the indicated bars.

stimulatory effect of PA on ATF4 and consequently upon SNAT2 gene expression.

4.2.12 PA may repress the SNAT2 adaptive response via ceramide

Although PA reduced the adaptive increase in SNAT2 transport activity and the abundance of the mature “heavy” SNAT2 protein, it most likely does so through a mechanism that differs from that utilised by LOA. Data in the literature show that PA and LOA exert opposite effects on the ubiquitin-proteasome pathway (Ando et al., 2004; Pauloin et al., 2010). To assess whether this is the case in my experimental model, L6 myotubes were incubated with 300 μM LOA or PA for 24 h and amino acid deprived for the last 4 h of this period in presence of MG132. As shown earlier, the intensity of the ubiquitin smear observed in presence of MG132 was enhanced in myotubes treated with LOA, while PA provision does not promote accumulation of ubiquitinated proteins, suggesting that its suppressive effect on System A transport is unlikely to involve SNAT2-degradation *via* the UPS (Fig 4.18). The inhibitory effect that PA exerts upon SNAT2 function might be partially mediated by its derivative ceramide, which has been previously shown to promote a reduction in SNAT2 activity by increasing internalisation of surface SNAT2 transporters, without significant loss in total cell SNAT2 content in L6 myotubes (Hyde et al., 2005). This possible mechanism would be in line with the finding that PA reduces the abundance of the mature SNAT2 glycosylated form without affecting the intracellular pool of the transporter. However, unlike in cells that were treated with ceramide, I observed a reduction in total cellular SNAT2 protein abundance in amino acid depleted cells incubated with PA compared to those not exposed to the fatty acid (Fig 4.15A compare lanes 2 and 4). This would suggest that, not

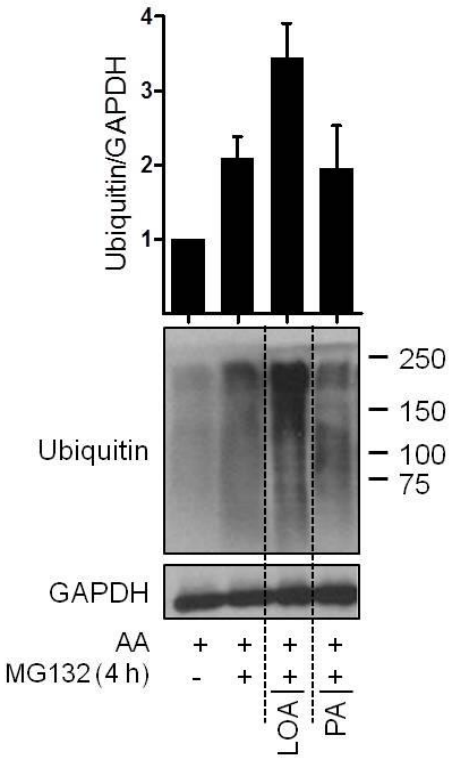


Figure 4.18 PA does not promote UPS activation.

L6 myotubes were incubated with EBSS media containing amino acids and 2% (w/v) BSA ± 300 µM linoleic acid (LOA) or palmitate (PA) for 24 h and treated with MG132 (10 µM) for the penultimate 4 h of this incubation as indicated. The immunoblots and relative quantifications are representative of two separate experiments.

withstanding the ability of ceramide to promote internalisation of SNAT2 from the cell surface, PA may target and direct the “surface” and internalised SNAT2 to non-proteasomal degradation, like, for example, lysosomal proteolysis. Moreover, it is important to consider that PA-induced ceramide production may be more pronounced in insulin target tissues, where these lipids have a major role as signaling factors influencing metabolic functions. If so, this would potentially explain why PA has an effect in L6 myotubes and not in HeLa cells. To test this hypothesis protein levels of LCB1, a subunit of the rate-limiting enzyme responsible for the *de novo* synthesis of ceramide from PA, serine palmitoyltransferase (SPT), were analysed in both cell lines. Fig 4.19A shows that LCB1 content was significantly higher in L6 myotubes than HeLa cells, suggesting that ceramide production was likely to be more pronounced in the skeletal muscle cell line. Ceramide involvement in mediating the PA suppressive effect was then tested by using myriocin, a well documented inhibitor of SPT and ceramide production. Fig 4.19B shows that the adaptive increase in the abundance of the “heavier” glycosylated SNAT2 was significantly reduced in L6 myotubes treated with PA for 24 h. However, when cells were co-incubated with PA and myriocin, the suppressive effect of the saturated fatty acid was significantly attenuated, indicating that ceramide synthesis was likely to be an important factor in the PA-induced reduction in cell surface SNAT2 expression and function (Fig 4.19B). As shown before, PA does not repress SNAT2 protein abundance in HeLa cells and this was not affected by co-provision of myrocin (Fig 4.19C).

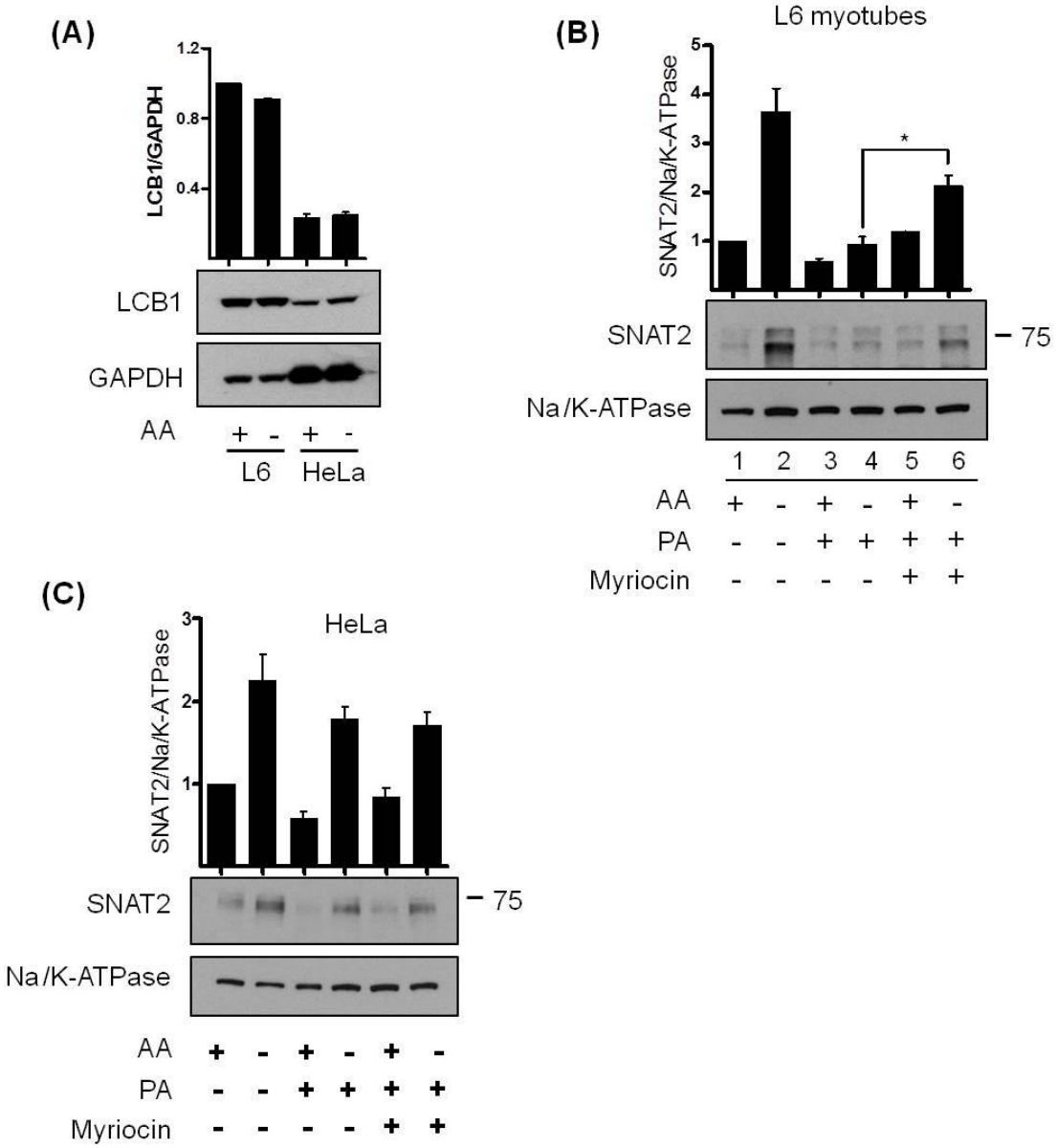

(A)
LCB1/GAPDH
1.2
0.8
0.4
LCB1
GAPDH
AA
+ - + -
L6
HeLa
(B)
L6 myotubes
SNAT2/Na/K-ATPase
5
4
3
2
1
*
SNAT2
75
Na/K-ATPase
1 2 3 4 5 6
AA + - + - + -
PA - - + + + +
Myriocin - - - - + +
(C)
HeLa
SNAT2/Na/K-ATPase
3
2
1
SNAT2
75
Na/K-ATPase
AA + - + - + -
PA - - + + + +
Myriocin - - - - + +

Figure 4.19 PA-induced loss of the mature "heavy" glycosylated SNAT2 protein involves ceramide production.

(A) L6 myotubes and HeLa cells were incubated with EBSS media containing amino acids (+) for 24 h and amino acid deprived (-) for the last 4 h of this incubation. **(B, C)** L6 myotubes (B) and HeLa cells (C) were incubated with EBSS media containing amino acids (+) and 2% (w/v) BSA ± 300 µM palmitate (PA) for 24 h and amino acid deprived (-) for the last 4 h. In some experiments cells were treated with myriocin (10 µM) for 4 h prior to and for the duration of the fatty acid incubation (*i.e.* total time of 28 h). Cell lysates (A) and isolated total membranes (B, C) were immunoblotted using antibodies against SNAT2 and NA/K-ATPase. Immunoblots are representative of three separate experiments. Data in the bar graphs is presented as mean ± SEM (n=3) with the asterisk indicating a significant difference between the indicated bars.

4.3 Discussion

4.3.1 LOA promotes SNAT2 degradation via proteasomal ubiquitin system

The work presented in this Chapter demonstrates that sustained cell incubation with LOA perturbs the adaptive upregulation of System A/SNAT2 that is normally instituted when cells are subjected to amino acid deprivation or a hypertonic-inducing stress stimulus. The upregulation of SNAT2 in response to such stimuli is not just a consequence of increased transcription of the *SLC38A2* gene (Palii et al., 2006) but also involves greater stabilization of the SNAT2 protein itself. However, my observations indicate that whilst the increase in SNAT2 gene expression triggered by extracellular amino acid deficiency or hypertonicity is unaffected, the resultant increase in SNAT2 protein and transport activity fail to materialise in cells preincubated with LOA because the transporter is targeted for degradation by the UPS. The ability of fatty acids to modulate SNAT2 abundance *via* this proteolytic route has not, to my knowledge, been previously documented and provides an additional facet to the biology of one of the most extensively regulated mammalian amino acid transporters known. However, it is important to stress that whilst the adaptive increase in SNAT2 abundance is diminished by LOA, the fatty acid also increases the total content of ubiquitinated proteins in cells (Fig 4.10A-C) suggesting that SNAT2 is likely to be one of many proteins targeted to the UPS in response to increased fatty acid availability. The ability of LOA to promote increased cellular protein ubiquitination and hence protein breakdown is likely to be matched by an ability to suppress protein synthesis given that the fatty acid also markedly reduced phosphorylation of P70S6K – a key upstream regulator of mRNA translation (Fig 4.10A and B). Thus a net consequence of LOA

oversupply to cells being subjected to amino-acid deprivation is accelerated loss in cellular protein, which may serve as a potential mechanism that helps maintain the intracellular amino acid pool during nutrient limited conditions. Precisely how unsaturated fatty acids, including LOA, stimulate the UPS remains unclear although in rat skeletal muscle they are known to stimulate the proteolytic activity of 20S proteasomes (Dahlmann et al., 1985). Accelerated skeletal muscle protein degradation is also a feature in mice fed a high fat diet in which enhanced *in vivo* muscle proteolysis has been linked to increased expression of two E3 ubiquitin ligases, atrogin-1/muscle atrophy F-box (MAFbx) and muscle RING finger-1 (MuRF-1) (Zhou et al., 2007), that are closely associated with muscle protein degradation (Sandri et al., 2004). Although there is no evidence in the literature linking MAFbx or MuRF-1 to regulation of SNAT2 turnover, I postulated that Nedd4.2 may be a prime candidate in the fatty acid induced degradation of SNAT2 given that this E3 ligase has been implicated in the ubiquitination of the transporter in 3T3-L1 adipocytes (Hatanaka et al., 2006). However, my data indicates that whilst LOA induces upregulation of Nedd4.2 in amino acid deprived cells in a manner that would be consistent with the observed reduction in SNAT2 triggered by LOA, cellular depletion of Nedd4.2 (using shRNA) was unable to prevent the loss in SNAT2 protein caused by the fatty acid. This latter observation, along with the finding that I do not observe any net increase in SNAT2 abundance in cells lacking Nedd4.2 *vis a vis* control cells (*i.e.* those infected with the empty lentiviral vector), would imply that Nedd4.2 is dispensable with respect to regulation of SNAT2 turnover *via* the UPS, at least in the cells used in my study.

Ectopic expression of EGFP-labelled SNAT2 (*aka* ATA2) in 3T3-L1 adipocytes has been used as a strategy for investigating SNAT2 ubiquitination and turnover and it is noteworthy that proteasomal inhibition within these cells using MG132 induced marked perinuclear accumulation of the transporter (Hatanaka et al., 2006). Intriguingly, in these cells MG132 also increased adipocyte Me-AIB uptake, which, coupled with the observation that Nedd4.2 partially colocalised with SNAT2 at the cell surface, led the authors of this study to conclude that surface SNAT2 would normally be internalised and degraded by the UPS (Hatanaka et al., 2006). Whilst there is some evidence that such a mechanism may account for the regulation and turnover of certain plasma membrane proteins (Malik et al., 2001; Smith et al., 2008; Zhao et al., 2014), most are internalised and undergo trafficking to early endosomes and sorting to multivesicular bodies prior to lysosomal fusion and proteolysis (Luzio et al., 2009; Piper and Katzmann, 2007). My studies assessing the effect of LOA on the SNAT2 adaptive response lead me to propose that SNAT2 may have two potential proteolytic fates depending on its cellular localization. It is my view that LOA targets the immature intracellular SNAT2 protein for degradation by the UPS, whereas the mature surface form is likely to be degraded by a non-UPS route (Fig 4.20). This proposition is based on the finding that MG132 not only curtails loss of immature SNAT2 ear-marked for degradation by LOA, but promotes its intracellular accumulation – much like that seen in adipocytes expressing EGFP-labelled SNAT2 (Hatanaka et al., 2006). However, since MG132 will have no direct inhibitory effect upon LOA-induced protein ubiquitination, I believe that the normal processing, maturation and delivery of SNAT2 to the cell surface (which represents an integral part of the System A

adaptive response) is likely to be arrested by its ubiquitination. Consistent with this view, MG132 fails to promote any buildup of the mature (cell surface) SNAT2 form in muscle cells and, as a consequence, System A transport activity cannot be raised to the level induced by amino acid deprivation or hypertonic stress as occurs in cells not treated with LOA and MG132 (Fig 4.11C and 4.11D). In addition, it is noteworthy that whilst Me-AIB uptake in cells maintained in an amino acid sufficient environment is normally low, LOA *per se* has no impact on basal amino acid uptake or upon the abundance of the mature/heavy SNAT2 protein although the fatty acid is still capable of causing a modest reduction in the abundance of the immature SNAT2 protein (compare lanes 1 and 3 in Fig 4.11A and 4.11B).

Previous work from the Hundal lab has indicated that the cytoplasmic domains of SNAT2 are the most likely candidate regions for associating with the proteolytic machinery and therefore most likely to confer amino acid-regulated stability effects (Hyde et al., 2007). Consistent with this idea, it has been shown that grafting the N-terminal tail of SNAT2 onto SNAT5 (a related family member that does not exhibit adaptation to amino acid withdrawal) renders the stability of the SNAT5 protein sensitive to amino acid availability (Hyde et al., 2007). Closer inspection of the cytoplasmic SNAT2 N-terminal tail reveals the presence of 7 lysine residues that may potentially serve as ubiquitin conjugation sites. The finding that transfection of HeLa cells with a SNAT2-V5 construct in which all 7 lysine residues were mutated to alanine results in expression of a SNAT2 protein that is constitutively more stable than wild-type SNAT2 and one that is protected against LOA-induced proteolysis would tend to support this proposition. A key unresolved issue concerns the identity of the E3-ligase that

promotes ubiquitination of one or more of these lysine residues and the mechanism by which LOA enhances this SNAT2-ubiquitin tagging process. Whilst increased expression of E3 ligases in response to unsaturated fatty acids may serve to drive ubiquitination/degradation of cellular proteins, the signalling events responsible for sensing and initiating this response remain poorly understood. However, two potential pathways that may contribute to fatty acid induced activation of the UPS include signalling initiated by TLR4, a member of the Toll-like family of receptors, and peroxisome proliferator-activated receptor α (PPARα), which functions as a major regulator of lipid metabolism in tissues such as liver and muscle (Burri et al., 2010). TLR4 activation can be typically initiated by binding of lipopolysaccharide (LPS) but also by fatty acids (Schaeffler et al., 2009; Shi et al., 2006) and, as such, activation of the receptor by LPS has been linked to upregulation of both MAFbx and MuRF-1 and increased muscle atrophy by the UPS (Doyle et al., 2011). Whether TLR4 signalling accounts for LOA-induced SNAT2 ubiquitination is currently unknown, but in separate experiments performed in the Hundal lab it has been found that whilst treatment of L6 myotubes with LPS induces TLR4 signalling there are no significant changes in System A mediated Me-AIB uptake (data not shown). Although this latter finding tends to negate involvement of the TLR4 signalling axis as a specific regulator of SNAT2 turnover it does not discount the possibility that it may stimulate loss of other cellular proteins by the UPS. Much like TLR4, PPARα also binds a variety of fatty acid ligands, preferentially unsaturated fatty acids including LOA, and recent work has highlighted that activation of PPARα increases content of ubiquitinated proteins in rat skeletal muscle and stimulates proteolytic flux through the UPS with a consequential

increase in muscle atrophy (Ringseis et al., 2013). As with TLR4 activation, the increase in protein ubiquitination associated with ligand-induced activation of PPARα involves higher muscle expression of MAFbx and MuRF-1. Whilst the impact of PPARα activation upon SNAT2 turnover has not been directly investigated, it is noteworthy that increases in SNAT2 abundance in response to chronic insulin stimulation can be fully repressed by PPARα activation in human trophoblasts (Jones et al., 2010). Whether this PPARα-linked repression is due to enhanced proteasomal degradation of SNAT2 or the result of impaired insulin signalling events that participate in the upregulation of SNAT2 expression is currently unknown, but testing these possibilities represent important investigative goals of future work.

4.3.2 PA-induced repression of SNAT2 transport activity is mediated by ceramide

It is well documented that PA, the most common circulating saturated fatty acid, induces insulin resistance (Powell et al., 2004), ER stress (Wei et al., 2006) and pro-inflammatory signalling (Joshi-Barve et al., 2007; Macrae et al., 2013) in insulin target tissues, while unsaturated fatty acids do not exert these detrimental effects but rather can mitigate the effects of saturated fatty acids (Coll et al., 2008; Dimopoulos et al., 2006; Kwon et al., 2014; Lee et al., 2006; Macrae et al., 2013; Yuzefovych et al., 2010). However, little is known of how fatty acids impact on amino acid transport. Data presented in this Chapter show that PA (a SFA) and LOA (a PUFA) have a similar inhibitory effect upon the SNAT2 adaptation response, even though they do so through different molecular mechanisms. Both LOA and PA impair the adaptive upregulation of SNAT2 transport activity induced by amino acid deprivation. This suppressive

effect manifests itself at doses of PA as low as 100 μM and is most apparent after a chronic incubation of cells with the fatty acid. One possible mechanism by which PA may repress SNAT2 adaptation is by decreasing *SLC38A2* gene expression in response to amino acid withdrawal. Interestingly, however, I found that PA enhanced SNAT2 mRNA abundance regardless of whether L6 cells were amino acid deprived or not (Fig 4.16A). SNAT2 expression is promoted by stresses, such as amino acid deprivation or osmotic shock, and this effect is partially dependent upon ATF4, a transcription factor that plays an important role in the cellular stress response (Hundal and Taylor, 2009). PA is known to induce oxidative stress and ER stress in insulin target tissues (Green et al., 2011; Macrae et al., 2013; Salvado et al., 2013) and has been shown to increase ATF4 gene expression in hepatocytes (Wei et al., 2009; Wei et al., 2006). Since SNAT2 is an ATF4 gene target it is plausible that it might upregulate SNAT2 gene expression in response to stress. The finding that ATF4 mRNA was increased by PA treatment in the presence of amino acids is in line with this idea (Fig 4.16C). Moreover, co-provision of the MUFA oleic acid, which is known to antagonise PA-induced ER stress (Salvado et al., 2013), counteracts the stimulatory effect of PA on ATF4 gene expression and that on its gene target SNAT2 (Fig 4.17A and 4.17B), confirming the idea that PA upregulates amino acid transporter expression by promoting a cellular stress response. SNAT2, but not ATF4, mRNA levels were further enhanced when PA-treated cells were subject to amino acid deprivation. This might be due to the fact that the GCN2/ATF4 is not the only pathway regulating SNAT2 transcription in response to amino acid withdrawal. Previous work from the Hundal lab has in fact shown that JNK (a MAPK family member) is involved in

mediating the adaptive response in L6 myotubes (Hyde et al., 2007). c-Jun, a JNK substrate, has also been shown to bind the SNAT2 promoter (Palii et al., 2006). Since PA can induce pro-inflammatory signalling by activation of the MAPK pathways (Green et al., 2011), it is possible that the saturated fatty acid enhances SNAT2 gene expression by both activating ATF4 and JNK following amino acid depletion.

Althought treatment of L6 myotubes with PA increased the mRNA abundance of SNAT2 both in basal and amino acid deprived conditions, it was accompanied by a corresponding increase in protein expression only in cells held in amino acid containing media. The reduction in the adaptive increase of System A activity triggered by PA can in fact be attributed to a loss in the protein abundance of the mature/glycosylated form of SNAT2 during amino acid starvation. However, unlike LOA, which decreases content of both the “heavy” and “light” form of SNAT2, PA represses only the abundance of the mature form of the transporter while not affecting that of the intracellular form (Fig 4.15A). These results suggest that the repressive effect of PA on SNAT2 membrane transport activity is unlikely to be mediated by the same mechanism as that utilised by LOA, which targets the intracellular pool of SNAT2 for degradation. The notion that saturated and unsaturated fatty acids may mechanistically affect SNAT2 function by different mechanisms is further supported by data in the literature showing that PA and LOA exert opposite effects on the ubiquitin-proteasome pathway. LOA has been shown to increase ubiquitination and consequent degradation of the protein tyrosinase, while PA retards its proteolysis (Ando et al., 2004). The increased degradation of tyrosinase as well as SNAT2 is the result of a general enhancement in proteasomal activity

promoted by LOA, as evidenced by the significant accumulation of ubiquitinated proteins in L6 myotubes (Fig 4.10A) and cultured mouse melanoma cells (Ando et al., 2004) when co-incubated with the PUFA and MG132. On the contrary, in both cell lines PA treatment fail to promote an increase in ubiquitinated proteins in the presence of the proteasome inhibitor (Fig 4.18) (Ando et al., 2004).

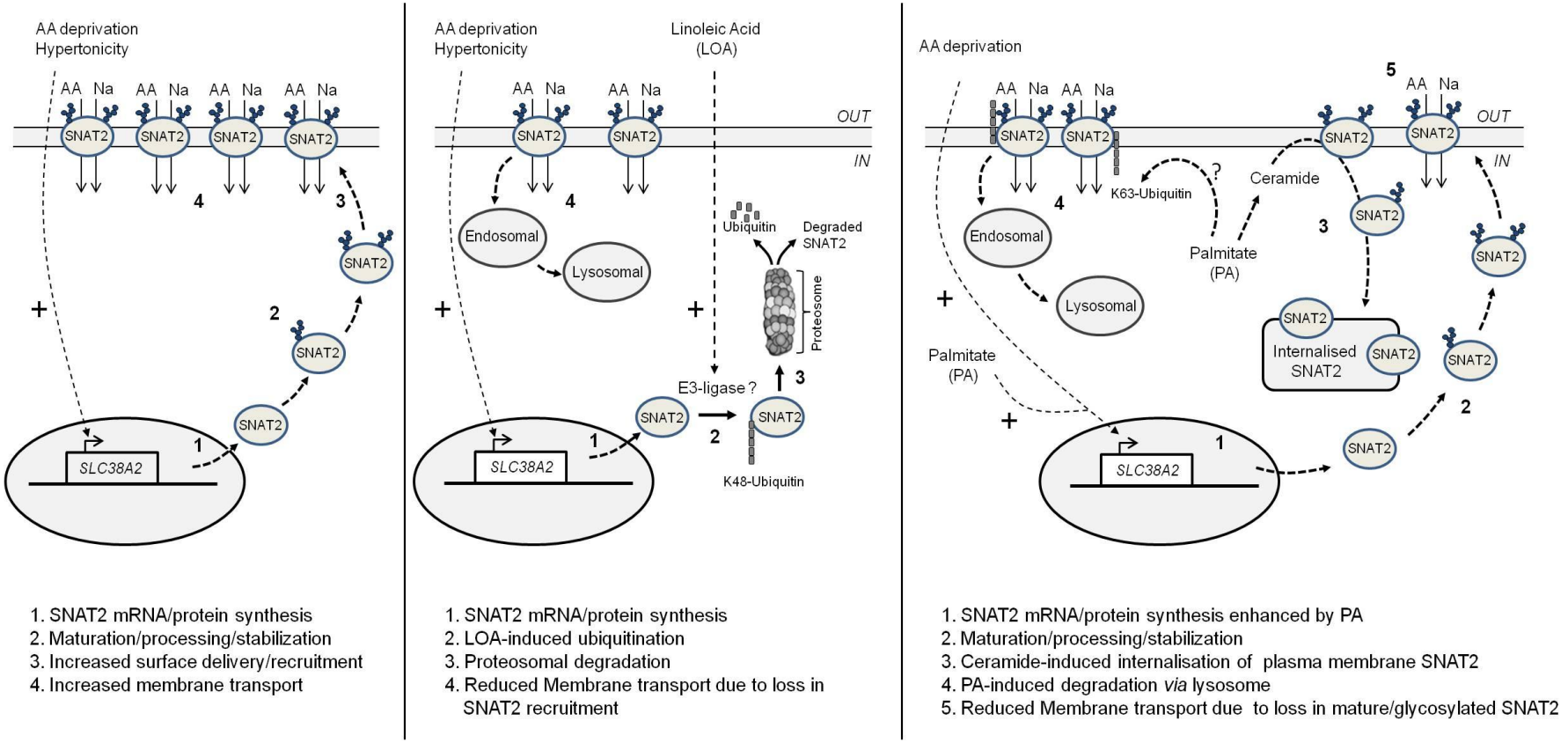
In the absence of any effects on the UPS, the inhibitory effect of PA on SNAT2 activity appears to be, in part, attributable to enhanced synthesis of its lipid derivative, ceramide. Previous work from the Hundal Lab has in fact shown that ceramide inhibits System A transport activity by enhancing internalisation of the transporter, thereby reducing the abundance of functional SNAT2 carriers at the cell surface (Hyde et al., 2005). One might assume that such internalisation is associated with the deglycosylation of the transporter, given that PA treatment preferentially results in accumulation of the “lighter” deglycosylated form of SNAT2. Although it is not possible to totally exclude ceramide involvement in LOA-induced repression of SNAT2 protein abundance, it is noterworthy that ceramide production from PUFAs is very restricted (Blachnio-Zabielska et al., 2010). Moreover, unlike LOA, which has no impact on protein abundance of the mature/glycosylated form of SNAT2 in amino acid sufficient conditions, ceramide has been shown to suppress both basal and insulin-stimulated System A activity as a result of depleting the plasma membrane abundance of SNAT2 (Hyde et al., 2005). This is in line with my finding, which shows that whilst PA upregulates SNAT2 expression, the fatty acid causes a modest reduction in the abundance of the mature “surface” SNAT2 protein (Fig 4.15A, compare lanes 1 and 3) and consequently in System A transport activity (Fig 4.14A). Ceramide involvement in mediating loss of plasma membrane SNAT2 in

response to PA was confirmed by the finding that myriocin, a well documented inhibitor of *de novo* fatty acid-derived synthesis of ceramide can partially prevent this loss in surface SNAT2 (Fig 4.19B, compare lanes 4 and 6). The inability of myriocin to completely restore protein levels of the mature/glycosylated form of SNAT2 following amino acid withdrawal may be explained by two possibilities. First, myriocin may not fully suppress SPT and consequently some *de novo* synthesis of ceramide still prevails. Moreover, amino acid depletion has been shown to increase ceramide production at least partly from sphingomyelin, a process catalysed by acid sphingomyelinase (SMase) activity, which is completely independent from SPT (Taniguchi et al., 2012). Second, it is plausible that, unlike ceramide, PA may induce degradation of the mature surface form of SNAT2. Whereas ceramide, generated from PA, may cause internalisation of SNAT2, the fatty acid may also target the mature/glycosylated form of SNAT2 for degradation by lysosome, since PA does not stimulate the ubiquitin-proteasome system. This proposition is supported by the finding that excess provision of SFAs in high-fat diets to mice (Guo et al., 2013) or exposure of pancreatic β cells to PA (Chen et al., 2013; Martino et al., 2012) enhances autophagic/lysosomal activity. Moreover, proteolysis induced by amino acid deprivation in skeletal muscle cells is mainly mediated by the lysosomal system (Bechet et al., 2005; Capel et al., 2009; Mordier et al., 2000). The ability of PA to modulate protein degradation *via* the lysosomal-proteolytic system is not unprecedented. In fact, the SFA has previously been shown to induce ER-stress and lipotoxicity in pancreatic β-cells by targeting carboxypeptidase E, the enzyme involved in the conversion of pro-insulin to insulin, for lysosomal degradation (Jeffrey et al., 2008). Thus, it is

possible that while ceramide promotes the internalisation of mature/ glycosylated form of SNAT2, PA might induce its K-63 linkage specific ubiquitination, which targets the mature “surface” form of the transporter for endocytosis and lysosomal degradation in cells subject to amino acid withdrawal (Fig 4.20).

In conclusion, the data presented in this Chapter show for the first time that LOA (a PUFA) and PA (a SFA) modulate System A transport activity by affecting SNAT2 protein stability. Both fatty acids exert a similar repressive effect upon the SNAT2 adaptive response, although they do so through distinct molecular mechanisms:

- LOA attenuates the adaptive upregulation of SNAT2 that is normally instigated in response to cellular amino acid depletion or hypertonic stress. This fatty acid-induced reduction is not due to suppression of SNAT2 gene transcription that is initiated by the amino acid withdrawal or hypertonic stimulus, but to enhanced destabilization and degradation of intracellular SNAT2 by the UPS. As a consequence, delivery of newly synthesised/processed SNAT2 to the cell surface is substantially diminished resulting in a concomitant loss in System A transport activity which will have implications for cellular adaptation to reduced extracellular amino acid availability and hypertonicity.
- PA reduces both basal and amino acid deprivation-induced SNAT2 transport activity. PA does not induce this decrease by suppressing ATF4 gene transcription or that of its target gene SNAT2, but rather *via* membrane depletion of the mature SNAT2 protein. Ceramide, a lipid derivative of PA, appears to mediate this effect by inducing



1. SNAT2 mRNA/protein synthesis
2. Maturation/processing/stabilization
3. Increased surface delivery/recruitment
4. Increased membrane transport

1. SNAT2 mRNA/protein synthesis
2. LOA-induced ubiquitination
3. Proteosomal degradation
4. Reduced Membrane transport due to loss in SNAT2 recruitment

1. SNAT2 mRNA/protein synthesis enhanced by PA
2. Maturation/processing/stabilization
3. Ceramide-induced internalisation of plasma membrane SNAT2
4. PA-induced degradation *via* lysosome
5. Reduced Membrane transport due to loss in mature/glycosylated SNAT2

Figure 4.20 Scheme illustrating the hypothetical mechanism of cellular regulation of SNAT2 in response to amino acid withdrawal or hypertonicity in the absence and presence of cell pretreatment with LOA and PA.

internalisation of mature/glycosylated form of SNAT2, while PA potentially targets the “surface” mature form of SNAT2 for lysosomal degradation in cells subjected to amino acid depletion.

Chapter 5

Conclusions and Future Work

5.1 Key observations from Chapter 3

- Oleic acid (OA, MUFA) and linoleic acid (LOA, A PUFA) enhance insulin-induced phosphorylation/activation of Akt and ERK1/2 and that of their downstream targets; this improved insulin action is not due to augmented IRS1/PI3K activation, but rather involves suppressed activation of protein phosphatase 2A (PP2A).
- OA and LOA mitigate the insulin-desensitising effects of palmitate (PA, a SFA) *via* counter-modulation of PP2A.
- An increase in fatty acid oxidation is not a requirement for promoting the protective effects of OA and LOA against PA-induced impairment of insulin signalling.
- The insulin-sensitising effects of OA and LOA do not involve modulation of AMPK nor do they rely upon the signalling-related functions of caveolin-enriched microdomains.

There is considerable scope for follow up work to try and further understand how MUFAs and PUFAs may potentially initiate and promote their effects upon insulin signalling. Three prospective avenues of research include (i) analysis of the role played by GPR40, a fatty acid receptor, (ii) changes in lipid handling/storage and (iii) effects upon mitochondrial biology. Possible experimental work in each of these areas is outlined below:

i. Does GPR40 mediate LOA and OA-induced inhibition of PP2A?

The data presented in Chapter 3 indicate that OA and LOA can oppose the insulin-desensitising effects of PA by countering the stimulatory effects that the SFA has upon PP2A. Tyrosine phosphorylation of the catalytic PP2A subunit on

Y307 is associated with its inhibition. Phosphorylation of this site is catalysed by the tyrosine kinase, Src, and given that this was enhanced in my studies with OA and LOA, my work would suggest that these fatty acids have the capacity to induce Src activation. However, the molecular mechanism by which OA and LOA induce Src activation remains unknown, but it is possible that it may involve activation of GPR40, a fatty acid receptor. GPR40 activates the Gαq/11 pathway, leading to release of Ca^{2+} from the ER (Burant, 2013), which has been associated with Src activation (El-Yassimi et al., 2008). Since unsaturated fatty acids, such as OA and LOA, have been shown to bind GPR40 (Briscoe et al., 2003) it would be of value to investigate its involvement in their beneficial effects by silencing expression of this receptor in L6 skeletal muscle cells and determine whether this negates the improvement in insulin sensitivity by UFAs.

Several studies have identified GPR40 as a key protein in the modulation of fatty acid-induced insulin secretion (Edfalk et al., 2008; Steneberg et al., 2005), thus in the past few years the receptor has become the focus of considerable interest as a novel therapeutic target for type 2 diabetes (Burant, 2013; Liu et al., 2014). Treatment of insulin resistant diabetic rodents with two of the most effective and potent GPR40 agonists identified so far, TAK-875 and AM 837, has been shown to increase glucose-stimulated insulin secretion and lower post-prandial blood glucose level (Lin et al., 2011; Tsujihata et al., 2011)., It would, therefore, be of interest to assess whether treating L6 myotubes with these agonists can mimic the insulin sensitising effect promoted by LOA and OA upon insulin signalling and, if so, whether this involves inhibition of protein phosphatase PP2A.

ii. TAG storage

Data presented in Chapter 3 indicate that fatty acid oxidation is not a requirement for promoting the beneficial effects of OA and LOA, given that etomoxir, an inhibitor of CPT-1 and therefore of fatty acid oxidation, did not diminish the ability of UFAs to enhance Akt activation and mitigate the detrimental effects of PA. This observation implies that OA and LOA may overcome the insulin desensitising effects of PA by channeling the fatty acid into neutral lipids (*i.e.* triglyceride), rather than increasing its oxidation. As discussed in section 1.6.3, increased partitioning and incorporation of SFAs towards TAG synthesis may lessen synthesis of other lipids, such as DAG and ceramides, implicated in insulin resistance (Cheon and Cho, 2014; Listenberger et al., 2003). Moreover, sequestering PA into TAG would be expected to reduce the carbon load placed on mitochondria thereby alleviating metabolic stress and promoting beneficial gains in insulin sensitivity. To explore whether increased TAG accumulation is a feature cells can be stained with oil red O, an oil soluble dye that accumulates within intramyocellular lipid droplets, as an initial approach to determine the amount of neutral lipid present in cells treated with PA alone or when coapplied with OA and LOA. TAG accumulation can also be quantitated using a radiometric assay in which the effects of UFAs upon incorporation of ^{14}C-palmitate into TAG can be determined. If UFAs do stimulate PA incorporation into neutral lipids then testing the involvement of Ubxd8 as a regulator of this process would be instructive. As discussed in Chapter 3, Ubxd8 acts as a key sensor of and mediator by which UFAs promote TAG synthesis (Lee et al., 2010). By binding Ubxd8, UFAs block Ubxd8-mediated degradation of Insig-1, a negative regulator of sterol regulatory element-binding protein 1

(SREBP-1) and consequently of fatty acid synthesis (Lee et al., 2010). Therefore, it would be interesting to determine the protein abundance of Insig-1 in L6 myotubes treated with PA alone or in combination with UFAs. One would postulate that Insig-1 would be degraded in cells incubated with the SFA but that OA and LOA would prevent this degradation. If so, it would be of value to explore whether silencing Ubxd8 expression in muscle cells would also stabilise Insig-1 expression and if this is able to improve TAG production/accumulation even in PA treated cells. This might mimic the protective effect associated with OA and LOA provision.

As mentioned above, binding of UFAs to Ubxd8 results in a decreased fatty acid synthesis *via* inhibition of SREBP-1. It is important to stress that down regulation of SREBP-1 and its target genes could itself have implications for the ability of UFAs to antagonise the repressive effect that PA exerts upon insulin signalling. This proposition is based on the finding that insulin resistance in humans and mice with primary hyperlipidemia (HPL) was correlated with increased level of SREBP-1 (Chu et al., 2013). This promoted elongation of PA to stearic acid, a process catalysed by very long-chain fatty acid elongase 6 (ELOVL6), a SREBP-1 target gene (Chu et al., 2013). Downregulation of SREBP-1 and ELOVL6 by small interfering RNA reduced serum stearic acid levels and ameliorated insulin resistance in HPL mice (Chu et al., 2013). In addition, knockdown of ELOVL6 has been shown to mitigate PA-induced ER stress and apoptosis in INS-1 β-cells (Green and Olson, 2011) while mice deficient in ELOVL6 on a high-fat diet display improved insulin sensitivity and are protected from hyperinsulinemia and hyperglycemia (Matsuzaka et al., 2007). Interestingly, several studies report that PUFA provision dramatically

suppresses ELOVL6 expression and improves insulin sensitivity in rodent hepatocytes and human colon epithelial cells (Howell et al., 2009; Narayanan et al., 2001; Sekiya et al., 2003). However, little is known about regulation of ELOVL6 by UFAs and how it may potentially help curb fatty acid-induced insulin resistance in skeletal muscle cells. Thus, it would be useful to establish whether PA upregulates expression of ELOVL6 and whether this increase can be antagonised when cells are coincubated with OA or LOA. If so, this study could be extended to assess whether treating L6 myotubes with ELOVL6 inhibitors, such as Compound A and B (Shimamura et al., 2010), could mimic the beneficial effects that OA and LOA have upon ameliorating PA-induced reduction in insulin signalling.

iii. Fatty acid regulation of mitochondrial function and morphology

In Chapter 3 I demonstrated the ability of OA and LOA to mitigate the insulin desensitising effects of PA *via* counter modulation of PP2A. However, it is highly likely that the mitigating effect of UFAs involves additional cellular mechanisms. There is mounting evidence to suggest that mitochondrial dysfunction and impaired oxidative capacity may play a significant role in SFA-induced insulin resistance in skeletal muscle (Bonnard et al., 2008; Hirabara et al., 2010; Koves et al., 2008; Muoio and Neufer, 2012; Turner and Heilbronn, 2008). Sustained incubation of muscle cells with PA is known, for example, to impair fatty acid oxidation, increase ROS production and decrease expression of proteins implicated in mitochondrial biogenesis, such as PGC1α and Tfam in muscle cells (Martins et al., 2012; Yuzefovych et al., 2010). Therefore, it is plausibile that the long-term benefits of UFAs on cellular function may depend upon preserving mitochondrial capacity and biogenesis. This could be assessed

via analysis of gene and protein expression of markers of mitochondrial biogenesis and function, such as PGC1α and COX 4.1, in muscle cells treated with LOA and OA alone or when coapplied with PA. Xue *et al.* demonstrated that the expression of SIRT1, a protein involved in the deacetylation and consequent activation of PGC1α, was increased by PUFAs in macrophages (Lagouge et al., 2006; Xue et al., 2012). Therefore, it would be of value to assess whether PGC1α is maintained in an acetylated/inactive state in cells treated with PA and whether cotreatment with OA and LOA promotes its deacetylation and activation. This could be assessed by immunoprecipitating PGC1α from L6 myotubes incubated with PA alone or with OA/LOA and immunoblotting with anti-acetyl lysine antibodies. If fatty acids do regulate PGC1α acetylation, the involvement of SIRT1 in mediating such modulation could be further confirmed through use of SIRT1 inhibitors such as X-527 and tenovin-1, or cells in which SIRT1 expression has been silenced using shRNA. One would predict that SIRT1 ablation would negate the beneficial effects associated with OA and LOA provision. Moreover, agonists of SIRT1 could be used to assess whether they mimic the insulin-sensitising effect of UFAs and antagonise PA-induced insulin resistance.

PA-induced mitochondrial dysfunction has been also linked to dysregulation of mitochondrial dynamics (Jheng et al., 2012). Mitochondria are dynamic organelles that constantly fuse and divide, events respectively called “fusion” and “fission”. Dynamin-related protein 1 (DRP1) regulates the fission process and has been shown to mediate PA-induced mitochodrial fragmentation and insulin resistance in skeletal muscle (Jheng et al., 2012; Watanabe et al., 2014). Therefore, it would be of interest to investigate whether the increase in DRP1

protein expression induced by PA can be prevented by cotreatment with OA and LOA. Moreover, it would be of value to assess whether the use of Mdivi-1, which inhibits DRP1 activity, mimics the beneficial effects promoted by UFAs upon insulin signalling in skeletal muscle cells. Studies performed in β-cells show that AMPK activation leads to phosphorylation of an inhibitory residue of DRP1, S637, preventing alterations in ER and mitochondrial morphology induced by PA (Wikstrom et al., 2013). Data presented in Chapter 3 indicate that although OA and LOA induce AMPK activation, this enhancement was dispensable for their insulin sensitising effect. However, whether AMPK is involved in mediating the ability of UFAs to antagonise the insulin-desensitising effect and, if so, whether this involves AMPK-mediated DRP1 inhibition remains unclear. This could be tested by assessing whether UFAs fail to attenuate the loss in Akt phosphorylation triggered by PA in AMPK-silenced L6 myotubes. If it is then the importance of DRP1 inhibition can be assessed using a specific phospho-antibody targeting the DRP1^{S637} residue in lysates from L6 myotubes treated with PA or UFAs. If UFAs do act as proposed above then one might predict that they may improve DRP1^{S637} phosphorylation when muscle cells are treated either alone with UFAs or when coapplied with PA.

5.2 Key observations from Chapter 4

- LOA represses adaptive System A/SNAT2 transport activity by targeting the immature “light” form of SNAT2 for degradation *via* the UPS.
- LOA-induced proteasomal loss of SNAT2 *via* the UPS does not involve the E3 ubiquitin ligase Nedd4.2.

- PA partially represses System A transport activity *via* ceramide synthesis.

There is considerable scope for further investigation of how UFAs and SFAs modulate the proteolytic machinery and more specifically SNAT2 stability. These include investigating (i) the identity and nature of the proteins that are sensitive to LOA-induced proteosomal degradation, (ii) the E3 ligase(s) responsible for LOA-induced SNAT2 ubiquitination, (iii) understanding more fully the mechanism by which PA regulates SNAT2 degradation and (iv) establishing the role played by the N-terminal SNAT2 tail in regulating its stability. These issues can be further explored by performing the types of experiments described below:

i. LOA-induced activation of UPS

The whole cell ubiquitin blots presented in Fig 4.9 and 4.10 indicate that SNAT2 is only one of many proteins ubiquitinated in response to LOA. As yet, the identity of these proteins and what cellular processes may be modulated as a consequence of their loss remains currently unknown. Therefore, a SILAC (stable isotope labeling with amino acids in cell culture) proteomic approach could be used to identify proteins sensitive to LOA-induced proteosomal degradation. This could be done, for example, by maintaining two identical myotube populations in custom media containing either 'light/natural' or 'heavy' forms of arginine (*i.e.* ^{12}C and ^{13}C labelled L-arginine, respectively). Cells will incorporate heavy arginine into newly synthesised proteins in the same manner as the normal “light” isotope. Unlabelled and labelled cells will be treated respectively with or without LOA for 24 h, and MG132 added to the media for

the last 4 h. Cells from both populations will then be pooled, proteins extracted and digested with trypsin. Ubiquitination occurs on the ε-amino group of lysine residues of target proteins; trypsin cleaves off all but the two C-terminal glycine residues of ubiquitin, which remain linked to the ε-amino group of the modified lysine residue. Peptides obtained from protein digests will be enriched by immunoprecipitation using a lysine-ε-GG antibody and peptide fragments subsequently analysed by *Liquid Chromatography–Mass Spectrometry (LC-MS)*. Sequence-identical peptides will be differentiated on the basis of mass difference and the ratio of peak intensities in the mass spectrum for such peptide pairs calculated to reflect the abundance ratio for the two peptides within the immunoprecipitate. This approach will identify which peptides become enriched in a LOA-dependent manner. The ms/ms spectra will then be searched against local databases using search engines (*e.g.* Mascot) to identify ubiquitinated proteins. This will allow classification/mapping of identified proteins to specific cell functions/pathways and follow-up studies on key candidate proteins for which commercial antibodies are available will help monitor/validate the effect LOA has upon their expression and/or function. This approach could provide new insights into the cellular processes regulated by fatty acid-induced modulation of the UPS.

ii. Which E3 ligase mediates LOA-induced SNAT2 ubiquitination?

Hatanaka *et al.* demonstrated that the E3 ubiquitin ligase Nedd4.2 regulates SNAT2 ubiquitination in 3T3-L1 adipocytes (Hatanaka et al., 2006). However, my data indicates that loss of SNAT2 protein induced by LOA is unlikely to involve this E3 ligase, since the ability of the fatty acid to promote SNAT2 degradation was not diminished in cells in which Nedd4.2 had been stably

depleted. Therefore, determining the identity of the E3-ligase that promotes LOA-induced SNAT2 degradation remains an unresolved issue. This issue could also be addressed using the SILAC proteomic approach described above, which would assess quantitative differences, at the protein level, between unlabelled and labelled cells treated or not with LOA for 24 h. This approach could help identify, among other proteins, which E3 ligases are up regulated by fatty acid treatment and, moreover, may also identify E2 and E1 ligases whose involvement and regulation by LOA cannot be excluded. qRT-PCR and western blot analysis could then be used to validate the differentially expressed E3 ligases quantified by SILAC. Their involvement in mediating the LOA-induced loss in SNAT2 protein could subsequently be assessed by silencing their expression in L6 myotubes and HeLa cells and asessesing whether this approach antagonises UPS-mediated SNAT2 degradation triggered by the fatty acid. The E3 ligases MAFbx and MuRF-1 remain potential candidates for mediating LOA-induced SNAT2 protein loss following amino acid deprivation. At present there is no evidence linking these E3 ligases to SNAT2 proteasomal degradation, but given that both have been implicated in proteolysis of muscle specific proteins (Bodine and Baehr, 2014; Zhou et al., 2007), their involvement in regulation of SNAT2 warrants further investigation. Therefore, it would be interesting to determine whether the SILAC-proteomic analysis discussed earlier identifies them as proteins upregulated by LOA treatment and, if so, whether suppressing their cellular expression mitigates the LOA-induced loss of SNAT2. If MAFbx and MuRF-1 is upregulated by LOA and can be linked to LOA-induced SNAT2 degradation then two possible molecular mechanisms may account for their upregulation. First, AMPK, which is activated in response

to stress-inducing conditions such as amino acid deprivation (Ghislat et al., 2012; Xiao et al., 2011) or hypertonic shock (Barnes et al., 2002; Sid et al., 2010), has been shown to increase MAFbx and MuRF-1 expression in C2C12 cells (Krawiec et al., 2007). My work, and that of others, has shown that unsaturated fatty acids enhance AMPK phosphorylation/activation (Salvado et al., 2013; Watt et al., 2006). Thus, it is plausible that LOA might induce these two E3 ligases as a consequence of stress-induced activation of AMPK. To test this hypothesis, the effect of LOA treatment upon the SNAT2 adaptive response triggered by amino acid deprivation or hypertonicity could be investigated in L6 myotubes in which AMPK has been stably silenced using shRNA. Another possible mechanism by which LOA could promote UPS activation is through activation of PPAR receptors such as PPARα. As indicated in Chapter 3 and 4, PPARα is a major transcriptional regulator of lipid metabolism in skeletal muscle (Burri et al., 2010) and can be activated by binding fatty acid ligands, such as LOA (Forman et al., 1997). Ringseis *et al.* have shown that treating rats with PPARα agonists results in upregulation of MAFbx and MurF-1 genes, even though their promoters are not directly regulated by the receptor (Ringseis et al., 2013). The involvement of PPARα in mediating LOA-induced up regulation of UPS could be explored by testing whether the fatty acid-induced loss of SNAT2 can be repressed by the use of PPARα antagonists, such as GW 6471 and MK 886, or PPARα gene silencing.

iii. Does PA induce lysosomal degradation of the mature “surface” form of SNAT2?

Data presented in Chapter 4 indicate that subjecting cells to PA treatment under conditions when they are being amino acid deprived results not only in a cellular

redistribution of the transporter, an event linked to PA-derived ceramide synthesis, but also a reduction in its total cellular content. Given that, unlike LOA, PA does not induce the UPS, it is plausible that it targets the mature form of SNAT2 for internalisation and degradation by the lysosome. Several membrane proteins are sorted and delivered to the lysosome in response to various stimuli in both yeast and mammalian cells (Ghaddar et al., 2014; Piper and Luzio, 2007). To test whether this is also the fate of the mature “surface” form of SNAT2, it would be of value to assess whether PA treatment increases accumulation of K63-ubiquitinated proteins in presence of a lysosomal inhibitor, such as leupeptin or chloroquine. While formation of K48-ubiquitin chains primarily directs proteins to the proteasome (Thrower et al., 2000), K63-ubiquitin chains target proteins mainly for lysosomal degradation (Ikeda and Dikic, 2008). If PA stimulates the lysosomal proteolytic system, it would then be of interest to investigate its effect upon the ubiquitination of the “surface” form of SNAT2. One possible strategy would involve using cell-impermeable reagents to biotinylate plasma membrane proteins. Biotin-labelled cell surface proteins will be then isolated by incubation with streptavidin agarose resins. The mature form of SNAT2 will be immunoprecipitated and probed for K-63 linkage specific ubiquitin. This strategy could be used in amino acid deprived cells treated with or without PA in presence of an endocytosis inhibitor, such as cytochalasin B (CB), so as to block internalisation of ubiquitin tagged SNAT2. One would expect that in presence of CB, PA treatment results in the accumulation of K-63 ubiquitinated mature “surface” form of SNAT2. PA-mediated internalisation and degradation of SNAT2 by the lysosome could be further validated by immunofluorescence analysis. It would be interesting to assess whether SNAT2

accumulates and colocalises with lysosomal markers in amino acid depleted L6 myotubes treated with PA and a lysosomal protease inhibitor, such as E64D. Moreover, SNAT2 protein loss at the plasma membrane induced by PA could be confirmed by performing immunofluorescence on non-permeabilised L6 myotubes using an antibody directed against an external (exo-facial) loop of the transporter.

iv. The N-terminal domain of SNAT2 is targeted by ubiquitin

Of the 12 lysine residues that may potentially be ubiquitinated on SNAT2, 7 are located within the SNAT2 N-terminal tail, making it highly likely that this transporter domain plays an important role in modulating transporter stability. This hypothesis was confirmed by the finding that in HeLa cells transiently expressing a SNAT2-V5 mutant, in which the 7 lysine residues had been mutated to alanine, the SNAT2 protein was more stable and resistant to LOA-induced proteasomal loss. However, it is still unknown which lysine residue(s) are selectively targeted for ubiquitination. Moreover, it is plausible that different lysines may be targeted by different type of ubiquitin chains, such as K48 and K63, thereby directing the protein to distinct proteolytic fates. To address this issue, it will be interesting to compare protein stability of different SNAT2-V5 mutants, in which the different lysine residues are systemically mutated to alanine with a view to identifying which mutated lysine residue(s) confer the greatest stability. Moreover, it would be of value to repeat this set of experiments in cells treated with either LOA or PA, in order to investigate which of the lysine residues are ubiquitinated under treatment with these fatty acids. It

will be possible to determine such residues as their mutation will prevent the loss in SNAT2 protein promoted by LOA or PA.

Identifying which lysines are ubiquitinated and which target the transporter for proteasomal or lysosomal degradation could help us understand why the mature and immature forms of SNAT2 protein have two different fates. It is plausible that, once at the plasma membrane, the mature “surface” form of SNAT2 would be subject to stimulus-induced conformational changes that may result in exposure of particular lysine residues that are targeted for K63-specific ubiquitination whereas these might not be accessible on the intracellularly localised protein. Recent studies have shown that, upon amino acid binding, yeast amino acid permeases shift to a conformation that favours their ubiquitination and consequent endocytosis. These structural changes involve the N-terminal tail and an intracellular loop of the permeases (Ghaddar et al., 2014). Whether this mechanism also holds for a mammalian amino acid transporter, such as SNAT2, is unknown but the concept is potentially testable by comparing protein stability of wild type (wt) SNAT2-V5 and the 7A-SNAT2-V5 mutant in HeLa cells or L6 myotubes subject to amino acid deprivation and then fed with a single SNAT2 amino acid substrate. One would expect that, upon amino acid binding, the wt SNAT2-V5 would be internalised and degraded by the lysosome whereas the 7A-SNAT2-V5 would be stable. This experiment can be repeated with the single lysine/alanine-SNAT2-V5 mutants to establish which lysine residue might be potentially targeted for K63 ubiquitination.

5.3 Concluding statement

Most international dietary guidelines suggest replacement of SFAs with MUFAs and PUFAs. Diets rich in UFAs have been shown to convey health benefits, including ameliorating the risk of metabolic syndrome and cardiovascular disease associated with SFA provision (Jones et al., 2014; Parillo et al., 1992; Summers et al., 2002). The work presented in this thesis provides novel insight into the beneficial effects that linoleic (a PUFA (LOA)) and oleic acid (a MUFA (OA)) confer against the adverse effects of the SFA palmitate (PA) in skeletal muscle. My studies show that these UFAs enhance insulin sensitivity and antagonise the detrimental effects of PA through counter modulation of PP2A in skeletal muscle. These findings are in line with previous work demonstrating that inhibition of PP2A restores insulin signalling in insulin-resistant ZDF rats (Galbo et al., 2011). In light of this evidence, PP2A activity can be considered as a valuable target for the treatment of type 2 diabetes and other metabolic disorders. Thus, the studies presented herein support the view that changing the dietary fatty acid composition in favour of UFAs may enhance insulin sensitivity in both normal and insulin-resistant skeletal muscle.

Interestingly, while increased PUFA intake is generally thought to bring about beneficial effects on health my results also demonstrate that LOA may compromise the function of molecules involved in nutrient uptake, such as the SNAT2 amino acid transporter. This effect is most evident under conditions of amino acid restriction and, moreover, is not limited to LOA but also observed in response to certain MUFAs and SFAs. Consequently, this fatty acid-induced effect may form part of a generic stress response that is triggered by nutrient limitation. During fasting, the body is metabolically challenged to maintain

energy homeostasis. After muscle and liver glycogen reservoirs have been depleted, triacylglycerol stores are broken down resulting in the release and subsequent rise in circulating free fatty acids. Indeed, recent studies have shown that the concentration of plasma free fatty acids increases from 0.19 (± 0.02) mmol/l to 0.76 (± 0.06) mmol/l in human subjects after 10 h of fasting (Wijngaarden et al., 2014). This increased lipolytic activity furnishes tissues such as muscle with fatty acids for use as metabolic fuel during fasting. However, prolonged periods of fasting will ultimately promote increased cellular proteolytic activity in which protein breakdown liberates amino acids, such as alanine, for use in hepatic gluconeogenesis and energy metabolism. There is evidence that certain fatty acids enhance the activity of the proteasomal or lysosomal proteolytic system (Dahlmann et al., 1985; Woodworth-Hobbs et al., 2014). Indeed, my studies indicate that LOA enhances protein breakdown through UPS induction and, furthermore, potentially decreases protein synthesis *via* modulation of kinases implicated in mRNA translation – such events would usually occur in the fasting state to maintain the free amino acid pool size. Therefore, it is plausible that free fatty acids, including LOA, released as a result of increased lipolysis may serve as a signal for improving the release of gluconeogenic precursors, such as alanine, *via* enhanced proteolysis by the UPS.

The SNAT2 adaptive phenomenon is a highly conserved cellular response that is initiated upon cells experiencing extracellular nutrient depletion. However, one might question the value of such response in the context of cellular adaptation to nutrient limitation given that provision of a single SNAT2 substrate

(in a backdrop when all other amino acids are still absent) is able to repress the SNAT2 adaptive response (Gazzola et al., 1981). Clearly, provision of a single amino acid will not be able to maintain the intracellular amino acid pool nor will it be sufficient for supporting protein synthesis. Consequently, the SNAT2 adaptation response might be considered as a conserved evolutionary mechanism that perhaps is now dispensable. The proteolytic process is highly selective in targeting non essential proteins for ubiquitination and avoids degrading proteins vital to the functioning of the cell. It is not surprising then, that SNAT2 falls within the proteins sensitive to LOA-induced proteasomal degradation. However, it is important to stress that, unlike an in vitro experimental setup, the physiological plasma amino acid pool during the fasting state is never completely depleted; therefore even a modest adaptive increase in SNAT2 abundance at the cell surface may enhance amino acid uptake. It is worth considering that glutamine and alanine, two of the main precursors of gluconeogenesis in small intestine and liver respectively (Garber et al., 1976), are also substrates of SNAT2. Therefore, LOA-induced inhibition of the transporter would reduce muscle tissue from extracting these amino acids from the circulation, which, instead, would be directed to gluconeogenic tissues, like the liver.

The ability of LOA to positively modulate glucose production in hepatocytes is not unprecedented (Suh et al., 2008). Indeed, it has been amply documented, both *in vivo* and *in vitro,* that free fatty acids improve gluconeogenesis (Chen et al., 1999; Gonzalez-Manchon et al., 1989; Morand et al., 1993; Williamson et al., 1966). Such enhancement has been linked to enhanced fatty acid oxidation.

During the fasting state fatty acids become the dominant fuel and their oxidation is increased in liver, resulting in enhanced level of acetyl-CoA generation. Acetyl-CoA allosterically activates pyruvate carboxylase, the enzyme catalysing the first committed step in gluconeogenesis (Lam et al., 2003). Thus, the studies presented herein suggest that during fasting state free fatty acids could stimulate glucose production not only by activating gluconeogenic enzymes, but also *via* increased provision of gluconeogenic precursors derived from breakdown of proteins in tissues such as muscle.

In conclusion, the work presented in this thesis has provided new insights into how fatty acids induce modulation of insulin signalling and nutrient uptake. The results indicate that provision of UFAs enhances insulin sensitivity and antagonise the deterimental effects of PA in skeletal muscle. However, my observations also suggest that, regardless of whether the fatty acid is saturated or unsaturated, free fatty acids released from lipid stores during the fasted state are likely to modulate the cellular ubiquitination machinery in order to enhance proteolysis, which, in turn, would be expected to promote release of amino acids that potentially could serve as substrates for hepatic gluconeogenesis.

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Appendix 1. Antibodies

Antibody	Dilution	Size (kDa)	Company
4G10 phospho tyrosine	1:5000		Millipore
Acetyl-CoA Carboxylase phospho S79	1:1000	280	Cell Signaling
B-Actin	1:2000	42	Sigma
Akt/PKB phospho S473	1:1000	60	Cell Signaling
Akt/PKB phospho T308	1:1000	60	Cell Signaling
Akt/PKB	1:1000	60	Cell Signaling
AMPK phospho T172	1:1000	62	Cell Signaling
AMPK α1	1:1000	62	Cell Signaling
Caveolin 1		20	DSTT
Caveolin 3		17	DSTT
CREB S133	1:1000	36	Cell Signaling
GAPDH	1:10000	37	Sigma
GSK3 phospho S9/21	1:1000	47	Santa Cruz
GSK3	1:1000	47	Santa Cruz
ERK1/2 phospho T202/Y204	1:1000	42/44	Cell Signaling
ERK1/2	1:1000	42/44	Cell Signaling
Flag	1:3000		Sigma
IRβ	1:1000	97	Upstate Biotechnology
IRS1	1:1000	130	Upstate Biotechnology
JNK T183/Y185	1:1000	46/54	Cell Signaling
Nedd4.2		120	DSTT
P70S6K phospho T389	1:1000	70	Cell Signaling
P70S6K	1:1000	70	Cell Signaling

p38 T180/Y182	1:1000	43	Cell Signaling
PI3K p85	1:1000	85	Upstate Biotechnology
PP2Ac demethylated	1:1000	37	Upstate Biotechnology
PP2A pospho Y307	1:2000	37	Abcam
PP2A (for IP)		37	DSTT
PTEN	1:1000	47	Santa Cruz
S6 S240/244	1:1000	32	Cell Signaling
Slc382 (SNAT2)	1:1000	40/60	MBL
Src Y416	1:1000	60	Cell Signaling
α-subunit Na^+/K^+ ATPase	1:500	100	DSHB
Ubiquitin	1:1000		Dako
Ubiquitin linkage specific K48	1:1000		Abcam
Ubiquitin linkage specifi K63	1:1000		Abcam
V5	1:5000		Life Technologies

Appendix 2. Buffers

Common buffers	Phosphate-buffered saline (PBS)	150mM NaCl 3mM KCl 10mM Na_2HPO_4 2mM KH_2PO_4
	Hepes-buffered saline (HBS)	20mM Hepes 140mM NaCl 16mM KCl 5mM $MgSO_4$ 1.3mM $CaCl_2$ pH 7.4
	Buffer I	20mM Hepes 250mM Sucrose 2mM EGTA 3mM Na Azide pH 7.4 (+ protease cocktails)
	Lysis buffer	50mM TRIS pH 7.4 0,27 M Sucrose 1mM Na-Orthovanadate pH 10 (inhibits tyrosine phosphatases) 1mM EDTA (chelates Mg^{2+} ions to inactivate protein kinases) 1mM EGTA 10mM Na-β-glycerophosphate 50mM NaF (inhibits serine/threonine phosphatases) 5mM Na-pyrophosphate

		1% Triton X-100 0.1% 2-Mercaptoethanol (+ protease cocktail)
	Protease cocktail	10μM E-64 (inhibits cysteine peptidases) 10μM Leupeptin (inhibits cysteine/serine/threonine peptidases) 1μM Pepstatin A (inhibits asprtyl proteases) 1 mM PMSF (inhibits serine proteases) 2μg/ml Aprotinin (inhibits trypsin and related proteolytic enzymes)
SDS-PAGE and immunoblotting buffers	Running Buffer	25mM Tris base 200mM Glycine pH 8.3 1%(w/v) SDS
	Transfer buffer	20mM Tris base 150mM Glycine 20%(v/v) Methanol
	Tris-buffered saline with Tween	10mM Tris HCl 150mM NaCl pH 7.6 0.2% (v/v) Tween
	Laemmli buffer	62.5mM Tris/HCl pH 6.8 10% (v/v) glycerol 0.01% (w/v) Bromophneol blue 2% (w/v) SDS
	Enhanced chemiluminescence (ECL)	100mM TRIS pH 8.5

	Solution 1	2.5mM luminal 396µM p-Coumaric acid
	ECL Solution 2	100mM TRIS ph8.5 5.63mM H_2O_2

Appendix 3. shRNA sequences

Sense and antisense strands are underlined, hairpin loop is indicated by italics.

Hairpin	shRNA Sequences
Cav-1 sh483	Oligo 1: *CCGG*AAGCGATTGGCAAGATATTCA*CTCGAG*TGAATATCTTGCCAATCGCTT*TTTTTG* Oligo 2: *AATTCAAAAA*AAGCGATTGGCAAGATATTCA*CTCGAG*TGAATATCTTGCCAATCGCTT
Cav-3 sh35	Oligo 1: *CCGG*GGATCATCAAGGACATTCACT*CTCGAGAG*TGAATGTCCTTGATGATCC*TTTTTG* Oligo 2: *AATTCAAAAA*GGATCATCAAGGACATTCACT*CTCGAG*AGTGAATGTCCTTGATGATCC
Ampk α1	Oligo 1: *CCGG*ATGAGTCTACAGCTATACCAA*CTCGAG*TTGGTATAGCTGTAGACTCATTTTTTG Oligo 2: *AATTCAAAAA*ATGAGTCTACAGCTATACCAA*CTCGAG*TTGGTATAGCTGTAGACTCAT

Scramble	Oligo 1: *CCGG*CCTAAGGTTAAGTCGCCCTCG*CTCGAG*CGAGGGCGACTTAACCTTAGG*TTTTTG* Oligo2: *AATTCAAAAA*ACCTAAGGAATTGTCGCCCTCG*CTCGAG*CGAGGGCGACTTAACCTTAGG
Nedd4.2	Oligo 1 *CCGG*AACAATCGAACCACAACTTGG*CTCGAG*CCAAGTTGTGGTTCGATTGTT*TTTTTG* Oligo 2 *AATTCAAAAA*AACAATCGAACCACAACTTGG*CTCGAG*CCAAGTTGTGGTTCGATTGTT

Appendix 4. Primer sequences

Target	Sense primer (5'-3')	Anti-sense primer (5'-3')
PP2Ac-rat	CAGCTGGTGATGGAGGGATA	TAGTCTGGGGTACGACGAGT
PME1-rat	CTGCTCTTGGCAGGTGTTGA	GTCGGATCAGGAAAGTGGCA
Actin-rat	CCGACAGGATGCAGAAGAAG	ATCCACATCTGCTGGAAGGTC
SNAT2-rat	GCAGCCGGAGAAGGATGATGAAC	GAAGAGGGCGGCAAGCAAATACA
SNAT2-human	GTGTTAATGGCTGTGACCCTGAC	GAGACTATGACGCCACCAACTGA
ATF4-rat	ACCCAAACCTTATGACCCACCTG	GCTGCTGTCTTGTTTTGCTCCATC
ATF4-human	GGTCAGTCCCTCCAACAACAGC	CCAACAGGGCATCCAAGTCG
Nedd4.2-rat	TCTGCCACGGACAACTACAC	CACAGGCCTGAGTTGGGATT
Nedd4.2-human	CTTAGTCATCCAGTGGAGATTTGTG	AGCAACTCCAGCTCATTTTCATC
GAPDH-all	TGGAAAGCTGTGGCGTGAT	GCTTCACCACCTTCTTGAT
pLKO.1	AATGGACTATCATATGCTTACCGT	CCAAAGTGGATCTCTGCTGTC
SNAT2-rat (insert vector V5-His6)	CCGCTCGAGGGCCAGTGC	GGCTCTAGAATGTCCGCCTGCAGAGGC